PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Nam et al.
Appl. No.	:	10/009,011
Filed	:	July 19, 2002
For	:	HCV/BVDV CHIMERIC GENOMES AND USES THEREOF
Examiner	:	Lucas, Zachariah
Group Art Unit	:	1648

DECLARATION UNDER 37 CFR 1.132 OF SUZANNE U. EMERSON, Ph.D.

I, Suzanne U. Emerson, Ph.D., do hereby declare:

1.   I am a named inventor of the above-identified application. A true and correct copy of my Curriculum Vitae is attached as Exhibit 1.

2.   We subsequently published the patent application as Nam et al., 2001, J Virol Methods 97: 113, which I understand is of record. Fig. 4 in Nam 2001 illustrates that infectious HCV/BVDV expressed core, E1, and E2 proteins of HCV:
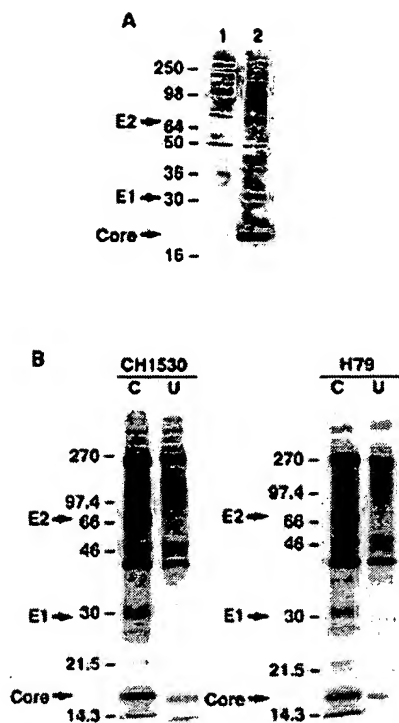
A Western blot (Fig. 4A) of cell lysate (lane 2) from EBTr(A) cells infected with HCV/BVDV showed immunoreactive proteins corresponding in size to core, E1, and E2 of HCV. A lysate of uninfected EBTr(A) cells (lane 1) did not contain these three bands. Also radioactively-labeled core, E1, and E2 of HCV were precipitated from a lysate of infected EBTr(A) cells by chimpanzee (CH1530) or human (H79) anti-HCV serum (Fig. 4B). These data demonstrate that the chimeric genome efficiently expressed the HCV structural proteins in infected EBTr(A) cells.

   3. Likewise, Fig. 5 in the patent specification, as described on p. 6, lines 2-5, p. 18, line 6 – p. 19, line 2, and p. 22, lines 26-29, illustrates that infectious HCV/BVDV expressed core, E1, and E2 proteins of HCV. These data reiterate that the chimeric genome efficiently expressed the HCV structural proteins in infected EBTr(A) cells.

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I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Respectfully submitted,

Dated: *August 9, 2005*   By: *Suzanne U. Emerson*
                                    Suzanne U. Emerson, Ph.D.

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EXHIBIT 1

CURRICULUM VITAE

Name: Suzanne Urjil Emerson, Ph.D.

Date and Place of Birth: July 1, 1943; Niagara Falls, New York

Citizenship: United States

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Education:

1965	B.S. (*cum laude*) - Biology, University of New Hampshire, Durham, New Hampshire
1965	Board Certified - Medical Technology Degree, Mary Hitchcock Hospital, Hanover, New Hampshire
1970	Ph.D. - Biology - University of California, San Diego, California. Ph.D. "Characterization of Bacterial Flagella", (advisor Melvin Simon)

Brief Chronology of Employment:

1965 - 1966	Medical Technologist, Robert B. Brigham Hospital, Boston, Massachusetts. (Performed all the usual laboratory assays and assembled and standardized the first Automated Analyzer for the chemistry division)
1970 - 1972	Postdoctoral Research Associate in laboratory of Robert R. Wagner, University of Virginia, Department of Microbiology
1972 - 1977	Assistant Professor, University of Virginia, Department of Microbiology
1977 - 1982	Associate Professor with tenure, University of Virginia, Department of Microbiology

1980	•	Sabbatical: Carnegie Institute of Embryology, Baltimore, MD
1982 - 1987		Professor, University of Virginia, Department of Microbiology
1986 - 1987		Visiting Research Scientist in Laboratory of Molecular Genetics, NINCDS
1988 - 1997		Microbiologist, Hepatitis Viruses Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892
1998-Present		Head, Molecular Hepatitis Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases

Societies:

Phi Beta Kappa
American Society for Microbiology
American Society for Virology
American Association for the Study of Liver Diseases

Honors, Awards and Medals Received:

1970 - 1972	U.S. Public Health Service Postdoctoral Fellowship
1973 - 1986	U.S. Public Health Service, Principal Investigator of Grant AI11722. "Rhabdovirus Transcription and Replication" (12/1/73-11/30/86). = $102,693 direct costs.
	U.S. Public Health Service, Research Career Development Award, A100013, "Functions and Biochemistry of Rhabdoviris Proteins"
	U.S. Public Health Service, Co-investigator, Program-Project Grant CA 18701, "Cancer Cell and Virus Membranes: Structure and Functions"
	U.S. Public Health Service, Co-director of Training Grant CA09109, "Cancer Research in Molecular Biology"
1975 - 1978	National Science Foundation - Member of Panel on Human Cell Biology
	National Institutes of Health - *ad hoc* Advisory, Virology Study Section

1978 - 1982	National Institutes of Health - Virology Study Section
	Site visits for ACS (Vanderbilt) NIH, (University of Alabama, Birmingham)
1987	American Cancer Society, Principal Investigator, "VSV RNA synthesis"
1995	Public Health Superior Service Award

Member of Editorial Board and/or Reviewer for the Following Journals:

1974 - 1995	Editorial Board Member: *Journal of Virology*
1977 - 1980	Editorial Board member: *Virology*
1989 - 1997	Editorial Board member: *Virology*

ad hoc Reviewer:
Journal of Clinical Microbiology
Journal of Medical Virology
Journal of Molecular Virology
Proceedings of the National Academy of Sciences, USA,
Virus Research
Hepatology
Lancet
Gastroenterology
Virology
Journal of Hepatology
Microbes and Infection
Journal of General Virology
Liver International
Journal Infectious Diseases

U.S. Patents:

Vaccine Against Hepatitis A Virus
Patent #: 4,894,228 (1990)
Inventors: Robert H. Purcell, John R. Ticehurst, Jeffrey I. Cohen, Suzanne U. Emerson, Stephen M. Feinstone, Richard J. Daemer, and Ian D. Gust

A Vaccine Against Hepatitis A
Patent #: 5,476,658 (1995)
Inventors: Sergei A. Tsarev, Suzanne U. Emerson, Michael S. Balayan, and Robert H. Purcell

Production of Complementary DNA Representing Hepatitis A Viral Sequences by
Recombinant DNA Methods and Uses Therefor
Patent #: 5,516,630 (1996)
Inventors: John R. Ticehurst, David Baltimore, Stephen Feinstone, Robert Purcell, Vincent
R. Racaniello, Bahige Baroudy, Suzanne U. Emerson

Production Of Complementary DNA Representing Hepatitis A Viral Sequences By
Recombinant DNA Methods And Uses Therefor
Patent #: 5,849,562 (Dec. 15, 1998)
Inventors: R.H. Purcell, S.U. Emerson

Recombinant Proteins of a Pakistani Strain of Hepatitis E And Their Use In Diagnostic
Methods And Viruses
Patent #: 6,054,567 (Apr. 25, 2000)
Inventors: Suzanne U. Emerson, Robert H. Purcell, Sergei A Tsarev, R.A. Robinson

Human/Simian Chimeric Hepatitis A Virus Vaccine
Patent #: 6,146,643 (Nov. 14, 2000)
Inventors: Sergei A. Tsarev, Suzanne U. Emerson, Robert H. Purcell

Cloned Genomes of Infectious Hepatitis C Viruses And Uses Thereof
Patent #: 6,153,421 (Nov. 28, 2000)
Inventors, M. Yanagi, J. Bukh, S.U. Emerson, R.H. Purcell

Methods For Detecting Antibodies to HAV 3C Proteinase
Patent #: 6,156,499 (Dec. 5, 2000)
Inventors: Deneen Stewart, Tina S. Morris, Robert H. Purcell, Suzanne U. Emerson

Attenuated Hepatitis A Virus Vaccine Which Grows in MRC-5 Cells
Patent #: 6,180,110 B1 (Jan. 30, 2001)
Inventors: Ann W. Funkhouser, Suzanne U. Emerson, Robert H. Purcell, Eric D'Hondt

Recombinant Proteins of a Pakistani Strain of Hepatitis E and Their Use in Diagnostic
Methods and Vaccines
Patent No. 6,207,416 (Mar. 27, 2001)
Inventors: Sergei A. Tsarev, Suzanne U. Emerson, Robert H. Purcell

Simian-Human HAV Having A Chimeric 2C Protein
Patent No. 6,280,734 (Aug. 28, 2001)
Inventors: G. Raychaudhuri, S.U. Emerson, R.H.Purcell

Recombinant Proteins of a Pakistani Strain of Hepatitis E and Their Use in Diagnostic
Methods and Vaccines
Patent No. 6,287,759 (Sept. 11, 2001)
Inventors: Sergei A. Tsarev, Suzanne U. Emerson, Robert H. Purcell

Vaccine Against Hepatitis A Virus
Patent #: RE37,381 (Sept. 18, 2001) of 4,894,228 (1990)

Inventors: Robert H. Purcell, John R. Ticehurst, Jeffrey I. Cohen, Suzanne U. Emerson, Stephen M. Feinstone, Richard J. Daemer, and Ian D. Gust

Hepatitis A Virus Vaccines
Patent #: 6,423,318 b1 (July 23, 2002)
Inventors: Ann W. Funkhouser, Suzanne U. Emerson, Robert H. Purcell, Eric D'Hondt

A Swine Hepatitis E Virus and Uses Thereof
Patent #: 6,432,408 B1 (Aug. 13, 2002)
Inventors: X-J Meng, S.U. Emerson, R.H. Purcell

Recombinant Proteins of a Pakistani Strain of Hepatitis E and Their Use in Diagnostic Methods and Vaccines
Patent #: US 6,458,562 B1 (Oct. 1, 2002)
Inventors: S.U. Emerson, R.H. Purcell, S.A. Tsarev, R.A. Robinson

Hepatitis A Virus Vaccines
Patent #: 6,680,060 B2 (Jan. 20, 2004)
Inventors: A.W. Funkhouser, S.U. Emerson, R.H. Purcell, E. D'Hondt

Recombinant Proteins of a Pakistani Strain of Hepatitis E and Their Use In Diagnostic Methods and Vaccines
Patent #: 6,696,242 (Feb. 24, 2004)
Inventors: S.A. Tsarev, S.U. Emerson, R.H. Purcell

Recombinant Proteins of a Pakistani Strain of Hepatitis E and Their Use In Diagnostic Methods and Vaccines
Patent #: USP 6,706,873 (Mar. 16, 2004)
Inventors: S.A. Tsarev, S.U. Emerson, R.H. Purcell

Special Scientific Recognition:

Invited Speaker and Chairperson, Symposium on Hepatitis E Virus: Epidemiology, Virology and Control of an Emerging Pathogen, New Delhi India, Feb. 18-19, 2005.

Invited Speaker, International Conference on Emerging Infectious Diseases, Al Ain, United Arab Emirates, Feb. 26-Mar. 1, 2005.

Invited Keynote Speaker, Australian Center for HIV and Hepatitis Virology Research, Terrigal,Aust., May 4-6, 2005.

BIBLIOGRAPHY

1. **Emerson SU**, Toluyasu K, Simon MI. Bacterial flagella: polarity of elongation. *Science* 1970;**169**:190-2.

2. **Emerson SU**, Simon MI. Variation in the primary structure of *Bacillus subitilis* flagellins. *J Bacteriol* 1971;**106**:949-54.

3. **Emerson SU**, Wagner RR. Dissociation and reconstitution of the transcriptase and template activities of vesicular stomatitis B and T virions. *J Virol* 1972;**10**:297-309.

4. Kelley JM, **Emerson SU**, Wagner RR. The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralizing antibody. *J Virol* 1972;**10**:1231-5.

5. **Emerson SU**, Wagner RR. The L protein is required for *in vitro* RNA synthesis by vesicular stomatitis virus. *J Virol* 1973;**12**:1325-35.

6. Bishop DHL, **Emerson SU**, Flamand A. Reconstitution of infectivity and transcriptase activity of homologous and heterologous viruses: vesicular stomatitis (Indiana serotype), Chandipura, vesicular stomatitis (New Jersey serotype), and coccal viruses. *J Virol* 1974;**14**:139-44.

7. **Emerson SU**, Yu Y-H. Both NS and L proteins are required for *in vitro* RNA synthesis by vesicular stomatitis virus. *J Virol* 1975;**15**:1348-56.

8. Wagner RR, **Emerson SU**, Imblum RL, Kelley JM. Structure-function relationships of the proteins of vesicular stomatitis virus. In Mahy BWJ, Barry RD, eds. *Negative Strand Viruses*. London, Academic Press 1975;1-19.

9. **Emerson SU**. Vesicular stomatitis virus: structure and function of virion components. *Curr Top Microbiol Immunol* 1976;**73**:1-34.

10. Hunt DM, **Emerson SU**, Wagner RR. RNA temperature-sensitive mutants of vesicular stomatitis virus: L-protein thermosensitivity accounts for transcriptase restriction of group I mutants. *J Virol* 1976;**18**:598-603.

11. **Emerson SU**, Dierks PM, Parsons JT. *In vitro* synthesis of a unique RNA species by a T particle of vesicular stomatitis virus. *J Virol* 1977;**23**:708-16.

12. Simon MI, **Emerson SU**, Shaper JH, Bernard PD, Glazer AN. Classification of *Bacillus subtilis* flagellins. *J Bacteriol* 1977;**130**:200-4.

13. **Emerson SU**, Dierks PM, Parsons JT. RNA synthesis by a defective particle of vesicular stomatitis virus. In Mahy BWJ, Barry RD, eds. *Negative Strand Viruses*. London, Academic Press 1978;565-76.

14. Mellon MG, **Emerson SU.** L and NS binding to ribonucleoprotein in vesicular stomatitis virus. In Mahy BWJ, Barry RD, eds. *Negative Strand Viruses.* London, Academic Press 1978;293-300.

15. Mellon GG, **Emerson SU.** Rebinding of transcriptase components (L and NS proteins) to the nucleocapsid template of vesicular stomatitis virus. *J Virol* 1978;**27**:560-7.

16. Schubert M, Keene JD, Lazzarini RA, **Emerson SU.** The complete sequence of a unique RNA species synthesized by a DI particle of VSV. *Cell* 1978;**15**:103-12.

17. Hunt DM, Mellon MG, **Emerson SU.** Viral transcriptase. In Bishop DHL, ed. *Rhabdoviruses*, Vol. I. Boca Raton, CRC Press 1979;169-83.

18. Kingsford L, **Emerson SU.** Transcriptional activities of different phosphorylated species of NS protein purified from vesicular stomatitis virus and cytoplasm of infected cells. *J Virol* 1979;**33**:1097-105.

19. Kingsford L, **Emerson SU**, Kelley JM. Separation of cyanogen bromide cleaved peptides of the vesicular stomatitis virus glycoprotein and analysis of their carbohydrate content. *J Virol* 1980;**36**:309-16.

20. Keene JD, Thornton BJ, **Emerson SU.** Sequence-specific contacts between the RNA polymerase of vesicular stomatitis virus and the leader RNA Gene. *PNAS USA* 1981;**78**:6191-5.

21. Belle-Isle HD, **Emerson SU.** Use of a hybrid-infectivity assay to analyze *ts* mutants of the New Jersey serotype of vesicular stomatitis virus affected in RNA synthesis. *J Virol* 1982;**43**:37-40.

22. **Emerson SU.** Reconstitution studies detect a single polymerase entry site on the vesicular stomatitis virus genome. *Cell* 1982;**31**:635-42.

23. McGowan JJ, **Emerson SU**, Wagner RR. The plus-strand leader RNA of vesicular stomatitis virus inhibits DNA-dependent transcription of adenovirus and SV40 genes in a soluble whole cell extract. *Cell* 1982;**28**:325-33.

24. Pinney DF, **Emerson SU.** Identification and characterization of a group of discrete initiated oligonucleotides transcribed *in vitro* from the 3'-terminus of the N-gene of vesicular stomatitis virus. *J Virol* 1982;**42**:889-96.

25. Pinney DF, **Emerson SU.** *In vitro* synthesis of triphosphate-initiated N-mRNA oligonucleotides is regulated by the matrix (M) protein of vesicular stomatitis virus. *J Virol* 1982;**42**:897-904.

26. Green TL, **Emerson SU.** Effect of the B-Y phosphate bond of ATP on synthesis of leader RNA and mRNAs of vesicular stomatitis virus. *J Virol* 1984;**50**:255-7.

27.   **Emerson SU**. Rhabdoviruses. In Fields BN, Knipe DM, Melnick JL, Chanock RM, Roizman B, Shope RE, eds. *Virology*. New York, Raven Press 1985;1119-32.

28.   **Emerson SU**. Transcription of vesicular stomatitis virus. In Wagner RR, ed. *The Rhabdoviruses*. New York, Plenum Publishing Company 1987;245-69.

29.   **Emerson SU**, Schubert MH. Molecular basis of rhabdovirus replication. In Perez-Bercoff R, ed. *Molecular Basis of Viral Replication*. New York, Plenum Publishing Company 1987;255-76.

30.   **Emerson SU**, Schubert MH. Location of the binding domains for the RNA polymerase L and the ribonucleopcapsid template within different halves of the NS phosphoprotein of vesicular stomatitis virus. *PNAS USA* 1987;**84**:5655-9.

31.   Liang L, **Emerson SU**. *In vitro* synthesis of large RNAs by an unusual defective interfering particle of vesicular stomatitis virus. *J Virol* 1988;**62**:1795-802.

32.   Williams PM, Williamson KJ, **Emerson SU**, Schubert M. Deletion mapping analyses indicate epitopes for monoclonal antibodies to the NS phosphoprotein of VSV are linear and clustered. *Virology* 1988;**164**:176-81.

33.   Williams PM, Williamson KA, **Emerson SU**. Monoclonal antibodies to the NS protein of vesicular stomatitis virus inhibit initiation of transcripts *in vitro* and dissociate leader RNA from mRNA synthesis. *Virology* 1988;**167**:342-8.

34.   **Emerson S**, Rosenblum B, Feinstone S, Purcell R. Identification of the hepatitis A virus genes involved in adaptation to tissue culture growth and attenuation. In Chanock RM, Lerner RA, Brown F, Ginsberg H, eds. *Vaccines 89: Modern Approaches to New Vaccines Including Prevention of AIDS*. Cold Spring Harbor, Cold Spring Harbor Laboratory 1989;427-30.

35.   Cox EM, **Emerson SU**, Lemon SM, Ping L-H, Stapleton JT, Feinstone SM. Use of oligonucleotide-directed mutagenesis to define the immunodominant neutralization antigenic site of HAV. In Brown F, Chanock RM, Ginsberg H, Lerner RA, eds. *Vaccines 90: Modern Approaches to New Vaccines Including Prevention of AIDS*. Cold Spring Harbor, Cold Spring Harbor Laboratory 1990;169-73.

36.   Wychowski C, **Emerson SU**, Silver J, Feinstone SM. Construction of recombinant DNA molecules by the use of a single stranded DNA generated by the polymerase chain reaction: its application to chimeric hepatitis A virus/poliovirus subgenomic cDNA. *Nucleic Acids Res* 1990;**18**:913-8.

37.   **Emerson SU**, McRill C, Purcell R. Effect of 5' mutations on the host range of hepatitis A virus in cell culture. In Hollinger FB, Lemon SM, Margolis H, eds. *Hepatitis and Liver Disease*. Baltimore, Williams & Wilkins 1991;62-3.

38. **Emerson SU**, McRill C, Rosenblum B, Feinstone S, Purcell RH. Mutations responsible for adaptation of hepatitis A virus to efficient growth in cell culture. *J Virol* 1991;**65**:4882-6.

39. **Emerson SU**, Tsarev SA, Purcell RH. Biological and molecular comparisons of human (HM-175) and simian (AGM-27) hepatitis A viruses. *J Hepatology* 1991;**13**:S144-5.

40. Negro F, **Emerson SU**, McRill C, Bonino F, Craxi A, Gerin JL, Miller RH, Purcell RH. A polymerase-chain reaction-based assay for serum HDV RNA. In Gerin JL, Rizetto M, Purcell RH, eds. *The Hepatitis Delta Virus*. New York, Wiley-Liss, Inc. 1991;179-84.

41. Tsarev SA, **Emerson SU**, Balayan MS. Sequence of simian AGM-27 strain of hepatitis A virus determined from polymerase chain reaction (PCR). In Hollinger FB, Lemon SM, Margolis H, eds. *Hepatitis and Liver Disease*. Baltimore, MD: Williams & Wilkins 1991;64-5.

42. Tsarev SA, **Emerson SU**, Balayan MS, Ticehurst J, Purcell RH. Simian hepatitis A virus (HAV strain AGM-27: Comparison of genome structure and growth in cell culture with other HAV strains. *J Gen Virol* 1991;**72**:1677-83.

43. **Emerson SU**, Huang YK, McRill C, Lewis M, Purcell R. Mutations in both the 2B and 2C genes of hepatitis A virus are involved in adaptation to growth in cell culture. *J Virol* 1992;**66**:650-4.

44. **Emerson SU**, Huang YK, McRill C, Lewis M, Shapiro M, London WT, Purcell RH. Molecular basis of virulence and growth of HAV in cell culture. *Vaccine* 1992;**10**:S36-9. (as proceedings of International Symposium on Active Immunization Against Hepatitis A, January, 1992, Vienna, Austria).

45. **Emerson SU**, Lewis M, Govindarajan S, Shapiro M, Moskal T, Purcell RH. A cDNA clone of hepatitis A virus encoding a virulent virus: Induction of viral hepatitis by direct nucleic acid transfection of marmosets. *J Virol* 1992;**66**:6649-54.

46. **Emerson SU**, McRill C, Purcell R. Effect of 5' mutations on the host range of hepatitis A virus in cell culture. In Hollinger FB, Lemon SM, Margolis H, eds. *Hepatitis and Liver Disease*. Baltimore, Williams & Wilkins 1992;62-3.

47. Purcell RH, D'Hondt ED, **Emerson SU**, Binn L, Govindarajan S, Bradbury R. Inactivated hepatitis A vaccine: Protective efficacy in chimpanzees. *Vaccine* 1992;**10**:S148-51. (as proceedings of International Symposium on Active Immunization Against Hepatitis A, January, 1992, Vienna, Austria).

48. Tsarev SA, **Emerson SU**, Reyes GR, Tsareva TS, Legters LJ, Malik IA, Iqbal M, Purcell RH. Characterization of a prototype strain of hepatitis E virus. *PNAS USA* 1992;**89**:559-63.

49.   **Emerson SU**, Huang YK, Purcell RH. 2B and 2C mutations are essential but mutations throughout the genome of HAV contribute to adaptation to cell culture. *Virology* 1993;**194**:475-80.

50.   Tedeschi V, Purcell RH, **Emerson SU**. Partial characterization of hepatitis A viruses from three intermediate passage levels of a series resulting in adaptation to growth in cell culture and attenuation of virulence. *J Med Virol* 1993;**39**:16-22.

51.   Tsarev SA, **Emerson SU**, Tsareva TS, Yarbough PO, Lewis M, Govindarajan S, Reyes GR, Shapiro M, Purcell RH. Variation in course of hepatitis E in experimentally infected cynomolgus monkeys. *J Infect Dis* 1993;**167**:1302-6.

52.   Tsarev SA, Tsareva TS, **Emerson SU**, Kapikian AZ, Ticehurst J, London W, Purcell RH. ELISA for antibody to hepatitis E virus (HEV) based on complete open-reading frame-2 protein expressed in insect cells: Identification of HEV infection in primates. *J Infect Dis* 1993;**168**:369-78.

53.   Yin SR, Tsarev SA, Purcell RH, **Emerson SU**. Partial sequence comparison of eight new Chinese strains of hepatitis E virus suggests the genome sequence is relatively stable. *J Med Virol* 1993;**41**:230-41.

54.   Arankalle VA, Chadha MS, Tsarev SA, Sehgal A, **Emerson SU**, Risbud AR, Banerjee K, Purcell RH. Seroepidemiology of water-borne hepatitis in India and evidence for a third enterically-transmitted hepatitis agent. *PNAS USA* 1994;**91**:3428-32.

55.   Bryan JP, Tsarev SA, Iqbal M, Ticehurst J, **Emerson SU**, Ahmed A, Duncan J, Rafiqui AR, Malik IA, Purcell RH, Legters LJ. Epidemic hepatitis E in Pakistan: Patterns of serologic response and evidence that antibody to hepatitis E virus protects against disease. *J Infect Dis* 1994;**170**:517-21.

56.   **Emerson SU**, Lewis M, Govindarajan S, Shapiro M, Moskal T, Purcell RH. In vivo transfection by hepatitis A virus synthetic RNA. *Arch Virol* 1994;**9**:205-9.

57.   Funkhouser AW, Purcell RH, D'Hondt E, **Emerson SU**. Attenuated hepatitis A virus: Genetic determinants of adaptation to growth in MRC-5 cells. *J Virol* 1994;**68**:148-57.

58.   Funkhouser AW, Purcell RH, **Emerson SU**. Mutations in the 5' noncoding, 2C, and P3 regions of the genome increase the efficiency of hepatitis A virus growth in MRC-5 cells. In Brown F, Chanock RM, Ginsberg HS, Norrby E, eds. *Vaccines 94: Modern Approaches to New Vaccines Including Prevention of AIDS*. Cold Spring Harbor, Cold Spring Harbor Laboratory Press 1994;345-9.

59.   Mannucci PM, Gdovin SL, Gringeri A, Colombo M, Mele A, Schinaia N, Ciavarella N, **Emerson SU**, Purcell RH. Transmission of hepatitis A to patients with hemophilia by factor VIII concentrates treated with organic solvent and detergent to inactivate viruses. *Ann Intern Med* 1994;**120**:1-7.

60. Purcell RH, Mannucci PM, Gdovin S, Gringeri A, Colombo M, Mele A, Schinaia N, CiavarellaN, **Emerson SU**. Virology of the hepatitis A epidemic in Italy. *Vox Sang* 1994;**67**(suppl 4):2-7.

61. Tsarev SA, Tsareva TS, **Emerson SU**, Govindarajan S, Shapiro M, Gerin JL, Purcell RH. Successful passive and active immunization of cynomolgus monkeys against hepatitis E. *PNAS USA* 1994;**91**:10198-202.

62. Tsarev SA, Tsareva TS, **Emerson SU**, Yarbough PO, Legters LJ, Moskal T, Purcell RH. Infectivity titration of a prototype strain of hepatitis E virus (HEV) in cynomolgus monkeys. *J Med Virol* 1994;**43**:135-42.

63. Yin SR, Purcell RH, **Emerson SU**. A new Chinese isolate of hepatitis E virus: comparison with strains recovered from different geographical regions. *Virus Genes* 1994;**9**:23-32.

64. Arankalle VA, Tsarev SA, Chadha MS, Alling DW, **Emerson SU**, Banerjee K, Purcell RH. Age-specific prevalence of antibodies to hepatitis A and E viruses in Pune, India, 1982 and 1992. *J Infect Dis* 1995;**171**:447-50.

65. D'Hondt E, Purcell RH, **Emerson SU**, Wong DC, Shapiro M, Govindarajan S. Efficacy of an inactivated hepatitis A vaccine in pre- and postexposure conditions in marmosets. *J Infect Dis* 1995;**171**(S1):S40-3.

66. Ghabrah TM, Strickland GT, Tsarev S, Yarbough P, Farci P, Engle R, **Emerson S**, Purcell RH. Acute viral hepatitis in Saudi Arabia: seroepidemiological analysis, risk factors, clinical manifestations, and evidence for a sixth hepatitis agent. *Clin Infect Dis* 1995;**21**:621-7.

67. Harmon SA, **Emerson SU**, Huang YK, Summers DF, Ehrenfeld E. Hepatitis A viruses with deletions in the 2A gene are infectious in cultured cells and marmosets. *J Virol* 1995;**69**:5576-81.

68. Schultheiss T, **Emerson SU**, Purcell RH, Gauss-Muller V. Polyprotein processing in echovirus 22: a first assessment. *Biochem Biophys Res Commun* 1995;**217**:1120-7.

69. Shaffer DR, **Emerson SU**, Murphy PC, Govindarajan S, Lemon SM. A hepatitis A virus deletion mutant which lacks the first pyrimidine-rich tract of the 5' nontranslated RNA remains virulent in primates following direct intrahepatic nucleic acid transfection. *J Virol* 1995;**69**:6600-4.

70. Tsarev SA, Tsareva TS, **Emerson SU**, Purcell RH, Govindarajan S, Shapiro M, Gerin JL. Successful passive and active immunization of cynomolgus monkeys against hepatitis E. In: Chanock RM, Brown F, Ginsberg HS, Norrby E, eds. *Vaccines 95: Molecular Approaches to the Control of Infectious Diseases*. Cold Spring Harbor, Cold Spring Harbor Laboratory Press 1995;263-6.

71. Tsarev SA, Tsareva TS, **Emerson SU**, Rippy MK, Zack P, Shapiro M, Purcell RH. Experimental hepatitis E in pregnant rhesus monkeys: failure to transmit hepatitis E virus (HEV) to offspring and evidence of naturally acquired antibodies to HEV. *J Infect Dis* 1995;**172**:31-7.

72. **Emerson SU**, Tsarev SA, Govindarajan S, Shapiro M, Purcell RH. A simian strain of hepatitis A virus, AGM-27, functions as an attenuated vaccine for chimpanzees. *J Infect Dis* 1996;**173**:592-7.

73. Funkhouser AW, Raychaudhuri G, Purcell RH, Govindarajan S, Elkins R, **Emerson SU**. Progress towards the development of a genetically engineered attenuated hepatitis A virus vaccine. *J Virol* 1996;**70**:7948-57.

74. Heller T, Yin SR, Tsarev SA, Purcell RH, **Emerson SU**. False-positive serologic test resulting from a probable yeast infection in a chimpanzee. *Clinical and Diagnostic Laboratory Immunology* 1996;**3**:614-5.

75. Purcell RH, **Emerson SU**. Hepatitis A and E: molecular biology. In: Schmid R, Bianchi L, Blum HE, Gerok W, Maier KP, Stalder GA, eds. *Acute and Chronic Liver Diseases: Molecular Biology and Clinics* (Proceedings of the 87th Falk Symposium) 1996:3-11.

76. Tellier R, Bukh J , **Emerson SU**, Purcell RH. Amplification of the full-length hepatitis A virus genome by long reverse transcription-PCR and transcription of infectious RNA directly from the amplicon. *PNAS USA* 1996;**93**:4370-3.

77. Tellier R, Bukh J, **Emerson SU**, Miller RH, Purcell RH. Long PCR and its application to hepatitis viruses: amplification of hepatitis A, hepatitis B, and hepatitis C virus genomes. *J Clin Micro* 1996;**34**:3085-91.

78. Tsarev SA, Tsareva TS, **Emerson SU**, Govindarajan S, Shapiro M, Gerin JL, Robinson R, Gorbalenya AE, Purcell RH. Prospects for prevention of hepatitis E. In: Buisson Y, Coursaget P, Kane M, eds. *Enterically-Transmitted Hepatitis Viruses* (Proceedings of the International Symposium on Enterically-Transmitted Hepatitis Viruses, Paris, France, October 1995) La Simarre, Joué-lès-Tours, France 1996;373-83.

79. Yanagi M, Bukh J, **Emerson SU**, Purcell RH. Contamination of commercially available fetal bovine sera with bovine viral diarrhea virus: implications for the study of hepatitis C virus in cell cultures. *J Infect Dis* 1996;**174**:1324-7.

80. Yu M, Miller RH, **Emerson SU**, Purcell RH. A hydrophobic heptad repeat of the core protein of woodchuck hepatitis virus is required for capsid assembly. *J Virol* 1996;**70**:7085-91.

81. Bukh J, **Emerson SU**, Purcell RH. Genetic heterogeneity of hepatitis C virus and related viruses. In: Rizzetto M, Purcell RH, Gerin JL, Verme G, eds. (Proceedings of IX Triennial International Symposium on Viral Hepatitis and Liver Disease, Rome Ital., April 1996) Edizoni Minerva Medica, Turin 1997;167-75.

82. Chatterjee R, Tsarev S, Pillot J, Coursaget P, **Emerson SU**, Purcell RH. African strains of hepatitis E virus that are distinct from Asian strains. *J Med Virol* 1997;**53**:139-44.

83. **Emerson SU**. Hepatitis A virus: molecular basis for replication *in vitro* and virulence *in vivo*. In: Rizzetto M, Purcell RH, Gerin JL, Verme G, eds. (Proceeding of IX Triennial International Symposium on Viral Hepatitis and Liver Disease, Rome Ital., April 1996) Edizoni Minerva Medica, Turin 1997;19-21.

84. Forns X, Bukh J, Purcell RH, **Emerson SU**. How Escherichia coli can bias the results of molecular cloning: preferential selection of defective genomes of hepatitis C virus during the cloning procedure. *PNAS USA* 1997;**95**:13909-14.

85. Meng XJ, Purcell RH, Halbur PG, Lehman JR, Webb DM, Tsareva TS, Haynes JS, Thacker BJ, **Emerson SU**. A novel virus in swine is closely related to the human hepatitis E virus. *PNAS USA* 1997;**94**: 9860-5.

86. Morris TS, Frormann S, Shechosky S, Lowe C, Lall MS, Gauss-Muller V, Purcell RH, **Emerson SU**, Vederas JC, Malcolm BA. *In vitro* and *ex vitro* inhibition of hepatitis A virus 3C proteinase by a peptidyl monofluoromethyl ketone. *Bioorganic & Medicinal Chemistry* 1997;**5**:797-807.

87. Raychaudhuri G, Govindarajan S, Purcell RH, **Emerson SU**. Studies toward development of a live attenuated vaccine for HAV using chimeras between human (HM-175) and simian (AGM-27) strains of HAV. In: Rizzetto M, Purcell RH, Gerin JL, Verme G, eds. (Proceeding of IX Triennial International Symposium on Viral Hepatitis and Liver Disease, Rome Ital., April 1996) Edizoni Minerva Medica, Turin 1997; 627-30.

88. Tellier R, Bukh J, **Emerson SU**, Purcell RH. Amplification of the full-length hepatitis A virus by long RT-PCR and generation of infectious RNA directly from the amplicon. In: Rizzetto M, Purcell RH, Gerin JL, Verme G, eds. (Proceeding of IX Triennial International Symposium on Viral Hepatitis and Liver Disease, Rome Ital., April 1996) Edizoni Minerva Medica, Turin 1997;48-50.

89. Tsarev SA, Tsareva TS, **Emerson SU**, Govindarajan S, Shapiro M, Gerin JL, Robinson R, Purcell RH. Recombinant vaccine against hepatitis E. In: Rizzetto M, Purcell RH, Gerin JL, Verme G, eds. (Proceeding of IX Triennial International Symposium on Viral Hepatitis and Liver Disease, Rome Ital., April 1996) Edizoni Minerva Medica, Turin 1997;648-9.

90. Tsarev SA, Tsareva TS, **Emerson SU**, Govindarajan S, Shapiro M, Gerin JL, Purcell RH. Recombinant vaccine against hepatitis E: dose response and protection against heterologous challenge. *Vaccine* 1997;**15**:1834-8.

91. Yu M, Miller RH, **Emerson S**, Purcell RH. A hydrophobic heptad repeat of the core protein of woodchuck hepatitis virus is required for capsid assembly. In: Rizzetto M,

Purcell RH, Gerin JL, Verme G, eds. (Proceeding of IX Triennial International Symposium on Viral Hepatitis and Liver Disease, Rome Ital., April 1996) Edizoni Minerva Medica, Turin 1997;94-7.

92. Stewart D, Morris TS, Purcell RH, **Emerson SU**. Detection of antibodies to the nonstructural 3C proteinase of hepatitis A virus. *J Infect Dis* 1997;**176**: 593-601.

93. Yanagi M, Purcell RH, **Emerson SU**, Bukh J. Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *PNAS USA* 1997;**94**:8738-43.

94. Bukh J, Kim JP, Govindarajan S, Apgar CL, Foung SK, Wages J Jr., Yun AJ, Shapiro M, **Emerson SU**, Purcell RH. Experimental infection of chimpanzees with hepatitis G virus and genetic analysis of the virus. *J Infect Dis* 1998;**17**:855-62.

95. Forns X, **Emerson SU**, Tobin GJ, Mushahwar IK, Purcell RH, Bukh J. DNA immunization of mice and macaques with plasmids encoding hepatitis C virus envelope E2 protein expressed intracellularly and on the cell surface. *Vaccine* 1998;**17**:1992-2002.

96. Funkhouser AW, Schultz DE, Lemon SM, Purcell RH, **Emerson SU**. Hepatitis A virus translation is rate-limiting for virus replication in MRC-5 cells. *Virology* 1998;**254**:268-78.

97. Ghabrah TM, Tsarev S, Yarbough PO, **Emerson SU**, Strickland GT, Purcell RH. Comparison of tests for antibody to hepatitis E virus. *J Med Virol* 1998;**55**:134-7.

98. Meng XJ, Halbur PG, Haynes JS, Tsareva TS, Bruna JD, Royer RL, Purcell RH, **Emerson SU**. Experimental infection of pigs with the newly identified swine hepatitis E virus (swine HEV), but not with human strains of HEV. *Arch Virol* 1998;**143**:1405-15.

99. Meng XJ, Halbur PG, Shapiro MS, Govindarajan S, Bruna JD, Mushahwar IK, Purcell RH, **Emerson SU**. Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. *J Virol* 1998;**72**:9714-21.

100. Pina S, Jofre J, **Emerson SU**, Purrcell RH, Girones R. Characterization of a strain of infectious hepatitis E virus isolated from sewage in an area where hepatitis E is not endemic. *Appl Environ Microbiol* 1998;**64**:4485-8.

101. Raychaudhuri G, Govindarajan S, Shapiro M, Purcell RH, **Emerson SU**. Utilization of chimeras between human (HM-175) and simian (AGM-27) strains of hepatitis A virus to study the molecular basis of virulence. *J Virol* 1998;**72**:7467-75.

102. Robinson RA, Burgess WH, **Emerson SU**, Leibowitz RS, Sosnovtesa SA, Purcell RH. Structural characterization of recombinant hepatitis E virus ORF2 proteins in baculovirus-infected insect cells. *Protein Expression and Purification* 1998;**12**:75-84.

103. Yanagi M, St. Claire M, **Emerson SU**, Purcell RH, Bukh J. *In vivo* analysis of the 3' untranslated region of the hepatitis C virus after *in vitro* mutagenesis of an infectious cDNA clone. *PNAS USA* 1998;**96**:2291-5.

104. Yanagi M, St. Claire M, Shapiro M, **Emerson SU**, Purcell RH, Bukh J. Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious *in vivo*. *Virology* 1998;**244**:161-172.

105. Yu M, **Emerson SU**, Cote P, Shapiro M, Purcell RH. The GDPAL region of the pre-S1 envelope protein is important for morphogenesis of woodchuck hepatitis virus. *Hepatology* 1998;**27**:1408-14.

106. Arankalle VA, Paranjape S, **Emerson SU**, Purcell RH and Walimbe AM. Phylogenetic analysis of hepatitis E virus isolates from India (1976-1993). *J Gen Virol* 1999;**80**:1691-1700.

107. Forns X, Hegerich P, Darnell A, **Emerson SU**, Purcell RH. High prevalence of TT virus (TTV) infection in patients on maintenance hemodialysis: frequent mixed infectious with different genotypes and lack of evidence of associated liver disease. *J Med Virol* 1999;**59**:313-17.

108. Kabrane-Lazizi Y, Fine JB, Elm J, Glass GE, Higa H, Diwan A, Gibbs CJ Jr., Meng XJ, **Emerson SU**, Purcell RH. Evidence for widespread infection of wild rats with hepatitis E virus in the United States. *Am J Trop Med Hyg* 1999;**61**:331-5.

109. Kabrane-Lazizi Y, Meng X-J, Purcell RH, **Emerson SU**. Evidence that the Genomic RNA of hepatitis E virus is capped. *J Virol* 1999;**73**:8848-50.

110. Meng XJ, Dea S, Engle RE, Friendship R, Lyoo YS, Sirinarumitr T, Urairong K, Wang D, Wong D, Yoo D, Zhang Y, Purcell RH, **Emerson SU**. Prevalence of antibodies to the hepatitis E virus (HEV) in pigs from countries where hepatitis E is common or is rare in the human population. *J Med Virol* 1999;**59**:297-302.

111. Purcell RH, **Emerson SU**. Hepatitis E. In Mandell GL, Bennett JE, Dolin R, eds. *Principles and Practice of Infectious Diseases.* New York, Churchill Livingstone, fifth Edition 1999;1958-70.

112. Yanagi M, Purcell RH, **Emerson SU**, Bukh J. Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. *Virology* 1999;**262**:250-63.

113. Yanagi, M, St. Claire M, **Emerson SU**, Purcell RH and Bukh J. In vivo analysis of the 3' untranslated region of hepatitis C virus following *in vitro* mutagenesis of an infectious cDNA clone. *PNAS USA* 1999;**96**:2291-5.

114. Allander T, Forns X, **Emerson SU**, Purcell RH, Bukh J. Hepatitis C virus envelope protein E2 binds CD81 of tamarins. *Virology* 2000;**277**:358-367.

115. Forns X, Payette Pj, Ma X, Satterfield W, Eder G, Mushahwar IK, Govindarajan S, Davis HL, **Emerson SU**, Purcell RH, Bukh J. Vaccination of chimpanzees with plasmid DNA encoding the hepatitis C virus (HCV) envelope E2 protein modified the infection after challenge with homologous monoclonal HCV. *Hepatology* 2000;**32**:618-25.

116. Fix AD, Abdel-Hamid M, Purcell RH, Shehata MH, Abdel-Aziz F, Mikhail N, Sebai HE, Nafeh M, Habib M, Arthur RR, **Emerson SU**, Strickland GT. Prevalence of hepatitis E in rural Egyptian communities. *J Trop Med Hyg.* 2000;**62**:5199-523.

117. Forns X, Thimme R, Govindarajan S, **Emerson SU**, Purcell RH, Chisari FV, Bukh J. Hepatitis C virus lacking the hypervariable region 1 of the second envelope protein is infectious and causes acute resolving or persistent infection in chimpanzees. *PNAS USA* 2000:97(24):13318-13323.

118. Purcell RH, **Emerson SU**. Hepatitis E virus infection. *Lancet* 2000;**355**:578.

119. Romeo R, Hegerich P, **Emerson SU**, Colombo M, Purcell RH, Bukh J. High prevalence of TT virus (TTV) in naïve chimpanzees and in hepatitis C virus-infected humans: frequent mixed infections and identification of new TTV genotypes in chimpanzees. *J Gen Virol* 2000;**81**:1001-7.

120. Schofield DJ, Glamann J, **Emerson SU**, Purcell RH. Identification by phage display and characterization of two neutralizing chimpanzee monoclonal antibodies to the hepatitis E virus capsid protein. *J Virol* 2000:**74**:5548-55.

121. Allander T, **Emerson SU**, Engle RE, Purcell RH, Bukh J. A virus discovery method incorporataing Dnase treatment and its application to the identification of two bovine parvovirus species. *PNAS USA* 2001;**98**:11609-14.

122. Bukh J, Apgar CL, Govindarajan G, **Emerson SU**, Purcell RH. Failure to Infect Rhesus Monkeys with Hepatitis C Virus Strains of Genotypes 1a, 2a or 3a. *J Viral Hepat* 2001;**8**:228-231.

123. Bukh J, Forns X, **Emerson SU**, Purcell RH. Studies of hepatitis C virus in chimpanzees and their importance for vaccine development. *Intervirology* 2001;**44**:132-142.

124. **Emerson SU**, Purcell RH. Recombinant vaccines for hepatitis E. (review) *Trends in Mol Med* 2001;**7**(10):462-466.

125. **Emerson SU**, Zhang M, Meng XJ, Nguyen H, St. Claire M, Govindarajan S, Huang YK, Purcell RH. Recombinant hepatitis E virus genomes infectious for primates: importance of capping and discovery of a cis-reactive element. *PNAS USA* 2001;**98**(26):15270-15275.

126. Halbur PG, Kosorndorkbua C, Gilbert C, Guenette D, Potters MB, Purcell RH, **Emerson SU**, Toth TE, Meng XJ. Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. *J Clin Microbiol* 2001;39(3):918-923.

127. Hollinger B, **Emerson SU**. Hepatitis A Virus. In Knipe D, Howley P, Griffin D, Lamb R, Martin M, Roizman B, Strauss S, eds. *Fields Virology*, Fourth Edition. Philadelphia, Lippincott, Williams and Wilkins 2001;799-840.

128. Kabrane-Lazizi Y, **Emerson SU**, Herzog C, Purcell RH. Detection of antibodies to HAV 3C proteinase in experimentally infected chimpanzees and in naturally infected children. *Vaccine* 2001 Apr 6;19(20-22):2878-2883.

129. Kabrane-Lazizi Y, Zhang M, Purcell RH, Miller KD, Davey RT, **Emerson SU**. Acute hepatitis caused by a novel strain of hepatitis E virus strain most closely related to the United States strains. *J Gen Virol* 2001 Jul;82(Pt7):1687-1693.

130. Nam J, Bukh J, Purcell RH, **Emerson SU**. High-level expression of hepatitis C virus (HCV) structural proteins by a chimeric HCV/BVDV genome propagated as a BVDV pseudotype. *J Virol Methods* 2001 Sept; 97 (1-2):113-123.

131. Purcell RH, **Emerson SU**. Animal models of hepatitis A and E. (Review) *ILAR J* 2001;42(2):161-177.

132. Purcell RH, **Emerson SU**. Chapter 89, Hepatitis E virus. In Knipe D, Howley P, Griffin D, Lamb R, Martin M, Roizman B, and Straus S, eds. *Fields Virology*. Philadelphia, Lippincott, Williams and Wilkins 2001;3051-3061.

133. Zhang M, **Emerson SU**, Nguyen H, Engle RE, Govindarajan S, Gerin JL, Purcell RH. Immunogenicity and protective efficacy of a vaccine prepared from 53kDa truncated hepatitis E virus capsid protein expressed in insect cells. *Vaccine* 2001;20(5-6):853-857.

134. Zhang M, Purcell RH, **Emerson SU**. Identification of the 5' terminal sequence of the SAR-55 and MEX-14 strains of hepatitis E virus and confirmation that the genome is capped. *J Med Virol* 2001;65:293-295.

135. Bukh J, Forns X, Thimme R, Govindarajan S, Miller RH, **Emerson SU**, Chisari FV, Purcell RH. Analysis of the hypervariable region 1 of hepatitis C virus. In: Margolis HS, Alter MJ, Liang TJ, Dienstag JL, eds. *Viral Hepatitis and Liver Disease* (Proceedings of the 10th International Symposium on Viral Hepatitis and Liver Disease) Atlanta, International Medical Press 2002:330-335.

136. Bukh J, Thimme R, Govindarajan S, Forns X, Satterfield X, Eder G, Chang K-M, Yanagi M, **Emerson SU**, Chisari FV, Purcell RH. Monoclonal hepatitis C virus infection in chimpanzees. In: Margolis HS, Alter MJ, Liang TJ, Dienstag JL, eds. *Viral Hepatitis and Liver Disease* (Proceedings of the 10th International Symposium on Viral Hepatitis and Liver Disease), Atlanta, International Medical Press 2002:336-340.

137.   **Emerson SU**, Huang YK, Nguyen H, Brockington A, Govindarajan S, St. Claire M, Shapiro M, Purcell RH. Identification of VP1/2A and 2C as virulence genes of hepatitis A virus and demonstration of genetic instability of 2C. *J Virol* 2002;**76**(17):8551-8559.

138.   **Emerson SU**, Miller KD, Davey RT, Purcell RH, Kabrane-Lazizi Y. Acute hepatitis caused by a hepatitis E virus strain related to the swine strain in a traveler returning from Thailand. In: Margolis HS, Alter MJ, Liang TJ, Dienstag JL, eds. *Viral Hepatitis and Liver Disease* (Proceedings of the 10th International Symposium on Viral Hepatitis and Liver Disease), Atlanta, International Medical Press 2002:116-118.

139.   Engle RE, Yu C, **Emerson SU**, Meng X-J, Purcell RH. Hepatitis E virus (HEV) capsid antigens derived from viruses of human and swine origin are equally efficient for detecting anti-HEV by enzyme immunoassay. *J Clin Microb* 2002:**40**(12):4576-4580.

140.   Meng XJ, Wiseman B, Elvinger F, Duenette DK, Toth TE, Engle R, **Emerson SU**, Purcell RH. Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. *J Clin Micro* 2002 Jan;**40**(1):117-122.

141.   Purcell RH, **Emerson SU**. Hepatitis A virus pathogenesis and attenuation. Chapter 33. In Semler BL, Wimmer E, editors, *Molecular Biology of Picornaviruses*. Washington, DC, ASM Press 2002;415-425.

142.   Schofield DJ, Rohwer-Nutter P, **Emerson SU**, Purcell RH. Identification of a major antigenic site on the HEV ORF2 protein (capsid) comprising neutralization and non-neutralization epitopes. In Margolis HS, Alter MJ, Liang TJ, Dienstag JL, eds. *Viral Hepatitis and Liver Disease. Proceedings of the 10th International Symposium on Viral Hepatitis and Liver Disease.* Atlanta, International Medical Press, Ltd. 2002:113-115.

143.   Schofield DJ, Satterfield W, **Emerson SU**, Purcell RH. Four chimpanzee monoclonal antibodies isolated by phage display neutralize hepatitis A virus. *Virology* 2002;**292**:127-136.

144.   Zhang M, **Emerson SU**, Nguyen H, Engle R, Govindarajan S, Blackwelder WC, Gerin J, Purcell RH. Recombinant vaccine against hepatitis E: duration of protective immunity in rhesus macaques. *Vaccine* 2002;**20**:3285-3291.

145.   Allander T, **Emerson SU**, Purcell RH, Bukh J. Cloning of unknown virus sequences by Dnase treatment and sequence-independent single primer amplification. In: Weissensteiner T, Griffin HG, Griffin A, eds. *PCR Technology, Current Innovations, 2nd Edition.* Boca Raton, CRC Press 2003:337-344.

146.   Bartosch B, Bukh J, Meunier J-C, Granier C, Engle RE, Blackwelder WC, **Emerson SU**, Cosset F-L, Purcell RH. *In vitro* assay for neutralizing antibody to hepatitis C virus: Evidence for broadly conserved neutralization epitopes. *PNAS USA* 2003;**100**:14199-14204.

147. **Emerson S**, Purcell RH. Hepatitis E virus. Reviews in Medical Virology 2003:**13**(3):145-154.

148. Graff J, **Emerson SU**. Importance of amino acid 216 in nonstructural protein 2B for replication of hepatitis A virus *in vivo. J Med Virol* 2003;**71**:7-17.

149. Purcell RH, Nguyen H, Shapiro M, Engle RE, Govindarajan S, Blackwelder WC, Wong DC, Prieels J-P, **Emerson SU**. Pre-clinical immunogenicity and efficacy trial of a recombinant hepatitis E vaccine. *Vaccine*, 2003;**21**:2607-2615.

150. Sakai A, St. Claire M, Faulk K, Govindarajan S, **Emerson SU**, Purcell RH, Bukh J. The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. *PNAS USA* 2003;**100**:11646-11651.

151. Schofield D J, **Emerson SU**, Purcell RH. Four chimpanzee monoclonal antibodies that neutralize hepatitis A virus. *Drugs of the Future* 2003;**28**(2):137-142.

152. Schofield DJ, Purcell RH, Nguyen HT, **Emerson SU**. Monoclonal antibodies that neutralize HEV recognize an antigenic site at the carboxyterminus of an ORF2 protein vaccine. *Vaccine* 2003;**22**:257-267.

153. Tellier R, Bukh J, **Emerson SU**, Purcell RH. Long PCR amplification of large fragments of viral genomes. In: Bartlett JMS, Stirling D, eds. *Methods in Molecular Biology.* Totowa, Humana Press, Inc. 2003:167-172.

154. Tellier R, Bukh J, **Emerson SU**, Purcell RH. Long PCR methodology. In: Bartlett JMS, Stirling D, eds. *Methods in Molecular Biology.* Totowa, Humana Press, Inc. 2003:173-178.

155. Yu C, Engle RE, Bryan JP, **Emerson SU**, Purcell RH. Detection of immunoglobulin M antibodies to hepatitis E virus by class capture enzyme immunoassay. *Clin Diagn Lab Immun* 2003;**10**(4):579-586.

156. Bukh J, Nam J-H, Faulk K, **Emerson SU**, Purcell RH. Development of an infectious cDNA clone permits *in vivo* analysis of genetic elements of GF virus B. In Jilbert AR, Grgacic EVL, Vickery K, Burrell CJ, Cossart YE, eds. *Proceedings of the 11th International Symposium on Viral Hepatitis & Liver Disease.* Australian Centre for Hepatitis Virology 2004:364-365.

157. Bukh J, Sakai A, Yanagi M, Forns X, St. Claire M, **Emerson SU**, Purcell RH. In vivo analyses of genetic elements of hepatitis C following in vitro mutagenesis of an infectious clone (pCV-H77C). In Jilbert AR, Grgacic EVL, Vickery K, Burrell CJ, Cossart YE, eds. *Proceedings of the 11th International Symposium on Viral Hepatitis & Liver Disease.* Australian Centre for Hepatitis Virology 2004:385-386.

158. Bukh J, Thimne R, Satterfield WC, Meunier JG, Forns X, Yanagi M, **Emerson SU**, Spangenberg HC, Chang KM, Chisari FV, Purcell RH. Persistence of HCV after

homologous monoclonal re-challenge is associated with emergence of virus variants. In Jilbert AR, Grgacic EVL, Vickery K, Burrell CJ, Cossart YE, eds. *Proceedings of the 11*[th] *International Symposium on Viral Hepatitis & Liver Disease.* Australian Centre for Hepatitis Virology 2004:375-377.

159. **Emerson SU**, Nguyen N, Graff J, Stephany DA, Brockington A, Purcell RH. *In vitro* replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. *J Virol* 2004;78(9):4838-4846.

160. Emerson SU, Purcel, RH. Running like water - The omnipresence of hepatitis E. *N Engl J Med* 2004 Dec 2;**351**(23):2367-2368.

161. **Emerson SU**, Schofield D, Nguyen H, Zhou Y-H, Purcell RH. Identification of the neutralization site of HEV suggests that rat HEV represents a new serotype. In Jilbert AR, Grgacic EVL, Vickery K, Burrell CJ, Cossart YE, eds. *Proceedings of the 11*[th] *International Symposium on Viral Hepatitis & Liver Disease.* Australian Centre for Hepatitis Virology 2004:436-437.

162. Huang FF, Sun ZF, **Emerson SU**, Purcell RH, Shivaprasad HL, Pierson FW, Toth TE, Meng XJ. Determination and analysis of the complete genomic sequence of avian hepatitis E virus (avian HEV) and attempts to infect rhesus monkeys with avian HEV. *J Gen Virol* 2004;**85**:1609-1618.

163. Koshy R, Le S-Y, Maizel J, **Emerson SU**, Purcell RH, Ehrenfeld E. Hepatitis C virus IRES elements: are two better than one? In Jilbert AR, Grgacic EVL, Vickery K, Burrell CJ, Cossart YE, eds. *Proceedings of the 11*[th] *International Symposium on Viral Hepatitis & Liver Disease.* Australian Centre for Hepatitis Virology 2004:359-360.

164. Men R, Yamashiro T, Goncalvez AP, Wernly C, Schofield DJ, **Emerson SU**, Purcell RH, Lai C-J. Identification of chimpanzee Fab fragments by repertoire cloning and production of a full-length humanized immunoglobulin G1 antibody that is highly efficient for neutralization of dengue type 4 virus. *J Virol* 2004:**78**(9):4665-4674.

165. Purcell RH, Nguyen H, Shapiro M, Engle RE, Govindarajan S, Blackwelder WC, Wong DC, Prieels J-P, **Emerson SU**. Preclinical immunogenicity and efficacy trial of a recombinant hepatitis E vaccine. In Jilbert AR, Grgacic EVL, Vickery K, Burrell CJ, Cossart YE, eds. *Proceedings of the 11*[th] *International Symposium on Viral Hepatitis & Liver Disease.* Australian Centre for Hepatitis Virology 2004:433-435.

166. Sakai A, Thimme R, Spanngenberg HC, Govindarajan S, **Emerson SU**, Purcell RH, Chisari FV, Bukh J. Rapid emergence of virus variants in acute HCV with vigorous host cellular immune responses and different outcome. In Jilbert AR, Grgacic EVL, Vickery K, Burrell CJ, Cossart YE, eds. *Proceedings of the 11*[th] *International Symposium on Viral Hepatitis & Liver Disease.* Australian Centre for Hepatitis Virology 2004:412-414.

167. Zhou Y-H, Purcell RH, **Emerson SU**. An ELISA for putative neutralizing antibodies to hepatitis E virus detects antibodies to genotypes 1, 2, 3, and 4. *Vaccine* 2004;**22**(20):2578-2585.

168. Graff J, Nguyen H, Kasorndorkbua C, Halbur PG, St Claire M, Purcell RH, **Emerson SU**. *In vitro* and *in vivo* mutational analysis of the 3'-terminal regions of hepatitis E virus genomes and replicons. *J Virol* 2005;**79**(2):1017-1026.

169. Graff J, Nguyen H, Yu C, Elkins WR, St. Claire M, Purcell RH, **Emerson SU**. The open reading frame 3 gene of hepatitis E virus contains a cis-reactive element and encodes a protein required for infection of macaques. *J Virol* 2005;**79(11)**:6680-6689.

170. Huang YW, Haqshenas G, Kasorndorkbua C, Halbur PG, **Emerson SU**, Meng XJ. Capped RNA transcripts of full-length cDNA clones of swine hepatitis E virus are replication-competent when transfected into Huh7 cells and infectious when intrahepatically inoculated into pigs. *J Virol* 2005;**79**(3):1552-1558.

171. Meunier JC, Engle RE, Faulk K, Zhao M, Bartosch B, Alter H, **Emerson SU**, Cosset FL, Purcell RH, Bukh J. Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1. *PNAS USA* 2005;**102**(12):4560-4565.

172. Purcell RH, **Emerson SU**. Hepatitis E virus. Chapter 174. In: Mandell GL, Bennett JE, Dolin R., eds, *Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases*. 6th edition. Philadelphia, Elsevier 2005:2204-2217.

173. Zhou YH, Purcell RH, **Emerson SU**. A truncated ORF2 protein contains the most immunogenic site on ORF2: antibody responses to non-vaccine sequences following challenge of vaccinated and non-vaccinated macaques with hepatitis E virus. *Vaccine* 2005;**23**(24):3157-3165.

1. **Emerson SU**, Arankalle VA, Purcell RH. Thermal stability of hepatitis E virus. *J Infect Dis* 2005, in press.

2. Purcell RH, **Emerson SU**. Hepatitis E prevention. In: Thomas HC, Lemon S, Zuckerman A, eds., *Viral Hepatitis*. 3rd edition. Oxford, Blackwell Publishing Ltd. 2005:635-648, in press.

3. Purcell RH, **Emerson SU**. Hepatitis A: Natural history and experimental models. In: Thomas HC, Lemon S, Zuckerman A, eds. *Viral Hepatitis*. 3rd edition. Oxford, Blackwell Publishing Ltd. 2005:109-125, in press.

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Generation and Characterization of a Hepatitis C Virus NS3 Protease-Dependent Bovine Viral Diarrhea Virus

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Unique to pestiviruses, the N-terminal protein encoded by the bovine viral diarrhea virus (BVDV) genome is a cysteine protease (Npro) responsible for a self-cleavage that releases the N terminus of the core protein (C). This unique protease is dispensable for viral replication, and its coding region can be replaced by a ubiquitin gene directly fused in frame to the core. To develop an antiviral assay that allows the assessment of anti-hepatitis C virus (HCV) NS3 protease inhibitors, a chimeric BVDV in which the coding region of Npro was replaced by that of an NS4A cofactor-tethered HCV NS3 protease domain was generated. This cofactor-tethered HCV protease domain was linked in frame to the core protein of BVDV through an HCV NS5A-NS5B junction site and mimicked the proteolytic function of Npro in the release of BVDV core for capsid assembly. A similar chimeric construct was built with an inactive HCV NS3 protease to serve as a control. Genomic RNA transcripts derived from both chimeric clones, $P_{H/B}$ (wild-type HCV NS3 protease) and $P_{H/B(S139A)}$ (mutant HCV NS3 protease) were then transfected into bovine cells (MDBK). Only the RNA transcripts from the $P_{H/B}$ clone yielded viable viruses, whereas the mutant clone, $P_{H/B(S139A)}$, failed to produce any signs of infection, suggesting that the unprocessed fusion protein rendered the BVDV core protein defective in capsid assembly. Like the wild-type BVDV (NADL), the chimeric virus was cytopathic and formed plaques on the cell monolayer. Sequence and biochemical analyses confirmed the identity of the chimeric virus and further revealed variant viruses due to growth adaptation. Growth analysis revealed comparable replication kinetics between the wild-type and the chimeric BVDVs. Finally, to assess the genetic stability of the chimeric virus, an Npro-null BVDV (BVDV−Npro in which the entire Npro coding region was deleted) was produced. Although cytopathic, BVDV−Npro was highly defective in viral replication and growth, a finding consistent with the observed stability of the chimeric virus after serial passages.

The *Flaviviridae* family currently comprises three genera of single-stranded positive-sense RNA viruses: flaviviruses, pestiviruses, and hepaciviruses (36). *Bovine viral diarrhea virus* (BVDV) is a prototype virus in the genus *Pestivirus,* which also includes *Classical swine fever virus* (CSFV) and *Border disease virus.* The RNA genome of BVDV is one of the largest (12.5 kb) among members of the *Flaviviridae* family (8). Similar to hepatitis C virus (HCV), it consists of a long 5′ untranslated region (UTR) which contains an internal ribosomal entry site (IRES) for the translation of viral proteins (6, 15, 35). The single large open reading frame encodes a polyprotein of approximately 3,900 amino acids (8, 29) that is processed into at least 12 functional proteins (Npro-C-E^{rns}-E$_1$-E$_2$/p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B) by both host and viral proteases (10, 36, 49). The first virally encoded protein is a unique protease (Npro for N-terminal protease), responsible for the cleavage between Npro and the core protein (C) (38, 41). A study by Rümenapf et al. showed that Npro is a novel type of cysteine proteinase which required cysteine$_{69}$ for proteolytic activity (38). Interestingly, partial and complete replacement of the Npro protein by a ubiquitin or fusion with a chloramphenicol acetyltransferase in pestivirus genomes had been shown to produce viable viruses (32, 45). The resulting chimeric viruses were demonstrated to have growth properties similar to the wild-type viruses.

As one of the most characterized members of the *Flaviviridae* family, BVDV provides a good model system for HCV, a major etiologic agent for non-A non-B hepatitis (1, 7). It shares many important features with HCV. Both viruses utilize an IRES within the 5′ UTR, for the translation of the viral polyprotein (6, 15, 35). Furthermore, the viral NS3 proteases of both viruses require NS4A as a cofactor for polyprotein processing (11, 25, 42). The cytopathic and plaque-forming properties of BVDV in cell cultures allow rapid and quantitative analysis of viral replication and growth. The availability of infectious clones (28, 46, 49) provides opportunities for genetic manipulation to alter viral functions and to construct chimeric viruses. Indeed, a recent report by Frolov et al. found that the entire BVDV IRES could be replaced by HCV IRES. The resulting chimeric viruses relied on the HCV IRES for growth (15), which should allow the in vitro efficacy evaluation of HCV IRES inhibitors.

HCV infection is prevalent and a major global health issue. A recently completed population-based survey revealed that in the United States alone the overall prevalence of anti-HCV was 1.8%, corresponding to an estimated 3.9 million individuals infected by HCV nationwide. A total of 74% of these seropositive individuals tested positive for HCV RNA, indicating that an estimated 2.7 million persons were chronically infected (2). Currently, the combination of alpha 2b interferon and ribavirin (Rebetron; Schering Plough, Kenilworth, N.J.) has been shown to have clinical efficacy in only a proportion (<50%) of patients with chronic HCV infection (9, 26). Vaccine development has been hampered by the high immune evasion rate with poor or no protection against reinfection with

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a heterologous or homologous inoculum in chimpanzees (12, 40, 48). Development of small molecule inhibitors directed against specific viral targets has thus become the major focus of anti-HCV drug development.

Extensive characterization of the HCV NS3 serine protease (3, 11, 16, 19, 21, 24) has shed light in developing assays and identifying inhibitors of HCV. Major advances in the determination of crystal structures for NS3 protease have begun to delineate important features for the development of potent and specific anti-HCV inhibitors (23, 50, 51). Many high-throughput enzyme-based screening assays, targeting HCV NS3 serine protease, have been developed. Further development of potential inhibitors has to rely on a convenient and reliable cell-based assay system to demonstrate their antiviral efficacy. The lack of a bona fide cell culture system that permits HCV infection makes it a daunting task to evaluate the antiviral efficacy of candidate inhibitors prior to in vivo studies in animals and humans.

Several HCV NS3 protease-dependent chimeric viruses using the genetic backbones of Sindbis virus and poliovirus have been created, providing potential cell-based antiviral assays to evaluate the efficacy of candidate inhibitors against HCV protease (4, 13, 14, 18). Similar schemes were adopted to create these chimeric viruses in which HCV NS3 protease-containing genes were inserted and fused in frame to an essential viral protein through an HCV junction site cleavable by HCV NS3 protease. Failure to cleave the junction by a mutant protease or in the presence of any potent HCV NS3 protease inhibitors would render the unprocessed viral proteins unable to perform their designated functions for viral growth (4, 13, 18). However, genetic stability of such chimeric viruses with foreign gene inserts was a major issue since RNA viruses recombined at a high frequency (29, 47). Indeed, the HCV NS3 genes inserted in the Sindbis viral genome were quickly deleted during initial viral passages and the revertant viruses appeared rapidly and exhibited similar advantageous growth properties as the wild-type viruses (13). Although a second generation of chimeric Sindbis viruses was generated in which a second HCV NS3 cleavage site was created, these viruses were rather defective and unable to replicate at the normal physiological temperature (13). This would limit the development of animal models for in vivo testing of the protease inhibitors.

We describe here the generation of a chimeric BVDV in which the Npro coding region is replaced by that of an NS4A cofactor-tethered HCV NS3 protease. This tethered HCV protease domain is fused in frame with the BVDV core protein via an HCV NS5A-NS5B junction site. In this chimeric design, the normal proteolytic function of the Npro is substituted by that of the HCV NS3 serine protease. We demonstrated that viable and cytopathic chimeric viruses were produced. They had growth kinetics comparable to that of the wild-type BVDV and were stable during subsequent serial passages. Our results suggest that the development of a cell-based antiviral assay is feasible using the HCV NS3 protease-dependent BVDV chimeric virus for in vitro testing of potential HCV NS3 protease inhibitors.

MATERIALS AND METHODS

Bacterial strains, oligonucleotides, and plasmids. Bacterial strains JM109 (DE3) and XL1-Blue were purchased from Promega (Madison, Wis.) and Stratagene (La Jolla, Calif.), respectively. DNA oligonucleotides were purchased from Life Technologies (Gaithersburg, Md.). Expression vector, pET-28a, was purchased from Novagen, Inc. (Madison, Wis.). The full-length molecular clone (pVVNADL) of the cytopathic BVDV (NADL isolate) was described previously (46). The entire BVDV genome was subcloned into a medium-copy-number p15A vector, resulting in a molecular clone, NADLp15a cl.4, with improved stability.

Construction of plasmids for in vitro expression of HCV NS3 and BVDV core fusion proteins. A single-chain HCV NS3 protease domain (H77 isolate), in which the NS4A cofactor peptide (GSVVIVGRIVLS) was fused in frame to the N terminus of the protease domain (amino acids 3 to 181) through a linker tetrapeptide (GSGS), was engineered and described previously (43). The mutation at amino acid 139 (from serine to alanine) of HCV NS3 protease was generated by using the QuickChange mutagenesis kit (Stratagene). A DNA fragment encoding the NS4A-tethered HCV NS3 protease and BVDV core was generated by using the standard PCR method as follows. A 5' PCR primer containing an NdeI site and coding region of NS4A cofactor (amino acids 21 to 25) was designed as the forward primer. A 3' PCR primer covering the coding region of NS3 (amino acids 175 to 181) and bearing a BamHI site was engineered as the reverse primer. The resulting PCR amplification of an NS4A-tethered HCV NS3 protease cDNA fragment consisted of NdeI and BamHI sites on either termini. The BVDV core cDNA was isolated similarly by using a long 5' primer encompassing a BamHI site, the NS5A-NS5B junction site (GADTEDVVCC-SMSY) and the N-terminal 1 to 7 amino acids of the core, and a 3' primer covering the C-terminal 97 to 102 amino acids of the core and an EcoRI site. The resulting PCR fragments were digested with the appropriate restriction enzymes and cloned in between the NdeI and EcoRI sites of pET-28a vector via a three-way ligation. Plasmid pNS3-C encodes a fusion protein consisting of an N-terminal NS4A cofactor-tethered single-chain HCV NS3 protease domain, a C-terminal BVDV core and an HCV NS5A-NS5B junction site as a linker in the middle. The plasmid pNS3mt-C was constructed similarly with a mutant HCV NS3 protease (S139A). Sequences of all clones were confirmed by dideoxynucleotide sequencing using an Automated Sequencer (ABI377; Perkin-Elmer, Foster City, Calif.). The amino acid sequences corresponding to the junctions of the fusion proteins are shown (see Fig. 1 and 5).

In vitro transcription and translation. HCV NS3 protease and BVDV core fusion proteins were expressed from the plasmids pNS3-C and pNS3mt-C by using the in vitro transcription and translation system (Promega) and labeled with [^{35}S]methionine (Amersham-Pharmacia Biotech, Arlington Heights, Ill.). The transcription-translation reactions were terminated by mixing with 2× sample buffer, and the protein products were separated on a 10 to 20% polyacrylamide gradient gel and analyzed by autoradiography.

Construction of chimeric BVDV plasmid. The chimeric clone (P$_{H/B}$) was constructed by the overlapping-extension PCR method (27) and standard molecular cloning techniques. Briefly, the 5' UTR was amplified by PCR with a ClaI/T7 promoter attached to the 5' end and a 3' end at the seventh codon of BVDV Npro. The cDNA fragment encoding the NS4A-tethered HCV NS3-BVDV core fusion protein was amplified from pNS3-C or pNS3mt-C. A third fragment covering the first amino acid of BVDV E^{rns} and amino acid 347 of E2 was also isolated by PCR. These three PCR fragments were joined together by using the TaqPlus long PCR system from Stratagene. The resulting PCR fragment (3,684 bp) was purified and digested with ClaI and RsrII and cloned into the BVDV pVVNADL clone between ClaI and RsrII. Both wild-type and mutant HCV NS3 protease chimeric BVDV plasmids P$_{H/B}$ and P$_{H/B(S139A)}$ were constructed (Fig. 1).

Construction of Npro-null BVDV. The Npro-null deletion mutant clone was constructed by using a PCR method known as gene splicing via overlapping extension (22, 39). Precise deletion of the entire open reading frame of Npro was accomplished by this PCR method so that the N terminus of core was fused directly in frame with the start codon of the BVDV genome.

Cell culture and virus stock. Madin-Darby bovine kidney (MDBK) cells were obtained from the American Type Culture Collection (CCL-22). MDBK cells were propagated in Eagle modified minimal essential medium (EMEM) supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 1.5 g of sodium biocarbonate (Bio-Whitaker) per liter, and 10% heat-inactivated horse serum (HS; Sigma, St. Louis, Mo.). Cell cultures were maintained at 37°C with 5% CO$_2$. The wild-type BVDV was derived from the molecular clone, and the high-titer viral stock was amplified in MDBK cells (46).

Large-scale production of full-length genomic RNA by in vitro transcription. The chimeric HCV NS3 protease-dependent BVDV plasmid (P$_{H/B}$) was used as the template for PCR amplification to generate the linearized cDNA of the entire genome with a 5' T7 promoter. The resulting long PCR fragment contains the entire BVDV genome under the T7 promoter and ends with the authentic 3' terminus. Two micrograms of the cDNA was transcribed into RNA by using the T7-MEGAscript Kit from Ambion (Austin, Tex.), according to the manufacturer's protocol. The RNA transcripts were extracted by phenol-chloroform and ethanol precipitated. The integrity of RNA transcripts was determined by 0.8% agarose gel electrophoresis, and the RNAs were stored at −80°C.

Transfection of MDBK cells with chimeric RNA transcripts. The in vitro-transcribed RNAs from P$_{H/B}$ were transfected into MDBK cells by electroporation as described (17, 28). Briefly, 5 μg of RNA transcripts was mixed with 0.1 ml of the cell suspension (2 × 10^7 cells/ml) and pulsed twice with a Gene Pulser (set at 0.4 kV and 25 μF with infinite resistance) from Bio-Rad (Hercules, Calif.). The electroporated cell suspension was mixed with EMEM supplemented with 10% HS and plated on a T-75 (75-cm^2) tissue culture flask (Becton Dickinson, Franklin Lakes, N.J.). The virally induced cytopathic effect was carefully examined. The culture media containing the progeny viruses were collected at 3 to 4 days posttransfection and used to infect fresh MDBK cells. The mutant RNA
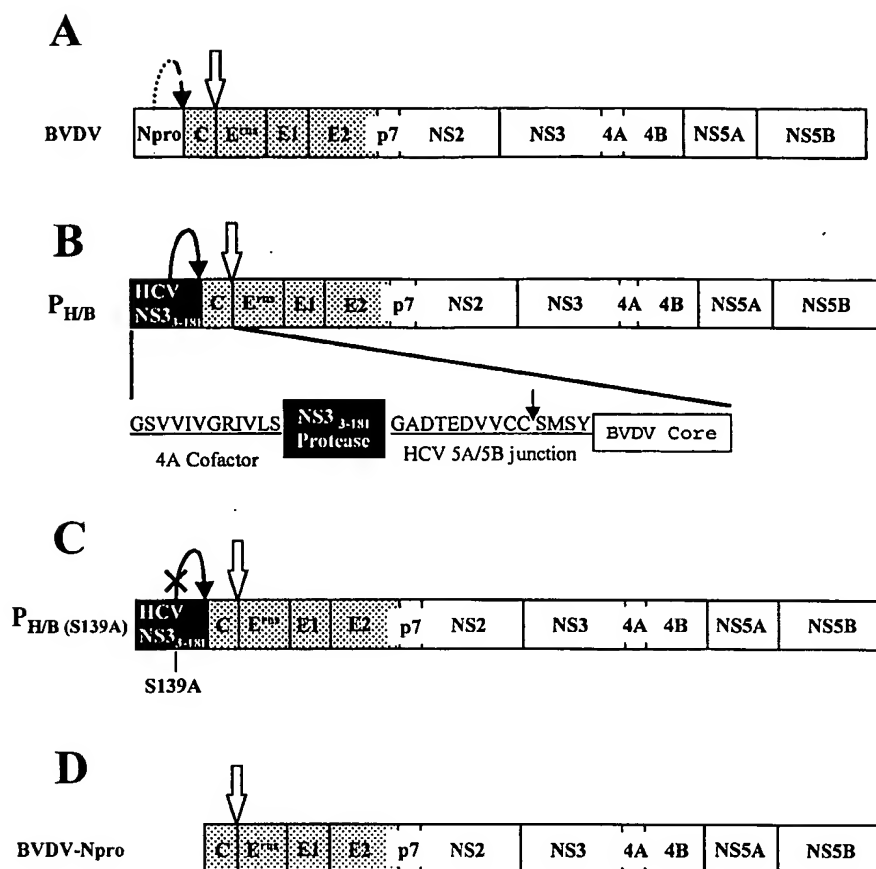
A

B

C

D

FIG. 1. Schematic design of chimeric HCV NS3 protease-dependent BVDV and Npro-null BVDV. (A) Genome organization of BVDV. (B and C) Genome structures of the HCV NS3 protease-dependent BVDV (P$_{H/B}$) and the mutant chimeric BVDV with an inactive HCV NS3 protease (P$_{H/B(S139A)}$), respectively. (D) Genome organization of BVDV−Npro. The shaded and open boxes represent BVDV structural and nonstructural polyproteins, respectively. The black boxes represent the HCV NS3 protease domain (residues 3 to 181). The open arrow indicates the cleavage site between the capsid (C) and E^{rns} of BVDV by host signal peptidase. The arrows with dotted and solid lines show the cis-cleavages of Npro of BVDV and HCV NS3 protease, respectively. Inactive HCV NS3 protease was generated by alanine substitution of serine 139 (vertical line with S139A). In panel B the amino acid sequences of the HCV NS4A, the NS5A-NS5B junction between HCV NS3 protease, and BVDV C are indicated by single-letter amino acid codes. The cleavage site of HCV NS3 protease is marked by a solid arrow.

transcripts from P$_{H/B(S139A)}$ were produced and transfected into MDBK cells in a similar manner.

Serial infections and generation of high-titer viral stocks. The chimeric viruses (V$_{H/B}$) produced by RNA transfection of MDBK cells were used to infect naive MDBK cells (5 × 10^6 cells plated in a T-75 tissue culture flask). After incubation at 37°C for 1 h, the inoculum was removed and replaced with 20 ml of fresh EMEM with 10% HS. The cells were incubated at 37°C for 2 days or until the cytopathic effect (CPE) was observed. The infection was repeated 10 times in MDBK cells using one-tenth of the previously infected culture media to infect the next plate of fresh MDBK cells. At each passage, extra culture media were either saved by storing at −80°C or used to infect more cells to amplify the virus stock.

Plaque assay and viral isolation. The plaque assay was described previously by Mendez et al. (28). The chimeric virus V$_{H/B}$ stocks were serially diluted in EMEM. The MDBK cells (0.5 × 10^6 cells seeded in each well of the six-well culture dish) were infected at 37°C with 0.5 ml of each dilution inoculum. After 1 h of adsorption, the inoculum was removed. The cell monolayer was overlaid with 1% low-melting-point agarose dissolved in EMEM containing 10% HS. The dishes were incubated for 3 days at 37°C. The monolayer of MDBK cells was fixed and stained with crystal violet staining solution containing 2.5% formaldehyde and 25% ethanol (37). Four well-separated plaques generated by the chimeric viruses were carefully removed with a pipette tip before fixation, and viruses in the agarose plugs were recovered in phosphate-buffered saline (PBS) at room temperature. The recovered viruses from each plaque were amplified in MDBK cells and used as the initial inoculum for serial passage of viral infection. A total of 10 passages were performed (P1 to P10), and viral stocks from each passage were collected and stored at −80°C.

One-step single cycle growth kinetic analysis. To determine the viral replication efficiency, MDBK cells (2 × 10^4 cells) in each well of a 24-well dish were infected with virus at a multiplicity of infection (MOI) of 5. After 1 h of incubation at 4°C, the inoculum was removed and the cell monolayer was washed with EMEM thoroughly to remove any unabsorbed viruses. Fresh EMEM with 10% HS was added, and the dish was incubated at 37°C. Media in individual wells were harvested at various time points as described in Results. The virus titers were determined by plaque assay and plotted against time to generate the one-step growth curves.

Isolation of viral RNA and RT-PCR analysis. Virus-containing culture medium (20 ml) was centrifuged at a low speed (10,000 rpm; Beckman SS34 Rotor) for 10 min at 4°C and then loaded onto a 10-ml sucrose cushion (30% in PBS). The viruses were pelleted by centrifugation at 25,000 rpm at 4°C for 10 h (Beckman SW28 Rotor). The virus pellet was resuspended in PBS and treated with RNase A (0.5 μg/μl) from Boehringer Mannheim (Indianapolis, Ind.) and RQ1-DNase I (1 U/μl) from Promega at 37°C for 3 h. Viral RNA was extracted by using phenol-chloroform and precipitated by ethanol in the presence of 0.2 M sodium chloride. Reverse transcription-PCR (RT-PCR) was performed on the viral RNA samples by using Thermoscript RT-PCR System from Life Technologies. The RT-PCR primers were as follows: 5'-GAGTACAGGACAGTCGTC AG-3' (forward primer corresponding to nucleotides 210 to 229 of the BVDV 5' UTR) and 5'-ACCAGTTGCACCAACCATG-3' (reverse primer complement ary to nucleotides 1620 to 1635 of the BVDV E^{rns}). Potential DNA contamina tion was assessed by PCR with omission of the RT step. Medium from mutant chimeric RNA (S139A) transfection was processed similarly, and RT-PCR was performed as described above. The RT-PCR yielded expected products of 1.4 kb. The PCR products were purified and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, Calif.) for direct sequencing and into the pET-28a vector for in vitro expression and cis-cleavage activity analysis of HCV NS3 protease-BVDV core fusion protein. All clones were verified by dideoxynucleotide sequencing by using an ABI377 Automated Sequencer.

Northern blotting analysis. Total intracellular RNA was prepared by using the NorthernMax Kit from Ambion (Austin, Tex.). Psoralen-biotinylated DNA probes derived from either the HCV NS3 protease or the BVDV NS5B were produced by using the BrightStar Psoralen-Biotin Labeling Kit (Ambion). Viral RNA was denatured by using glyoxal-dimethyl sulfoxide at 50°C for 30 min, separated by 1% agarose gel electrophoresis, and transferred onto the BrightStar Plus Membrane (Ambion). The membrane was incubated in hybridization solution at 42°C for 1 h, followed by overnight incubation in fresh hybridization solution supplemented with a 0.1 nM concentration of biotinylated DNA probes. The membrane was then washed, and the viral RNA was detected by using the BrightStar BioDetect Kit (Ambion).

Western blotting analysis. Cell lysates from infected cells were denatured and subjected to SDS-PAGE (on a 10 to 20% gradient gel) analysis. After electrophoresis, the proteins were electrotransferred onto a nitrocellulose membrane (Novex, San Diego, Calif.). The rabbit polyclonal antibodies raised against HCV NS3 protease and the monoclonal antibody raised against BVDV NS3 were used as the primary antibodies. Horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) and anti-rabbit IgG antibodies (Promega) were used as the secondary antibodies. The immunoreactive protein bands were detected by ECL Western Blot Detection Kit (Amersham-Pharmacia Biotech) and recorded on an X-ray film.

Nucleotide sequence accession number. The chimeric viral genome sequence was deposited in the GenBank database (accession no. AF268278).

RESULTS

Chimeric concept and experimental designs. HCV and BVDV are closely related (33). It is conceivable that the two viruses share similar replication strategies and regulatory interactions inside the host cells (36). Similar intracellular viral replication and assembly pathways may render BVDV a better choice to provide the genetic background for making HCV-dependent chimeric virus that may be superior to those viruses that are distantly related to HCV. Another advantage of using BVDV is its genomic plasticity (29), which allows recombinant manipulations with fewer concerns about genome compatibility. BVDV also consists of one of the largest genomes among members of the *Flaviviridae* family and encodes two unique viral proteins: Npro and E^{rns}. While the biological functions of these "extra" viral proteins are unclear, it has been shown that Npro can be deleted or replaced without significant effect on the viability and infectivity of BVDV and CSFV (30, 31, 44, 45). Taking advantage of the fact that the BVDV Npro protease is dispensable for viral replication and growth, its coding region was replaced by an NS4A cofactor-tethered HCV NS3 protease domain linked in frame to the BVDV core protein through an HCV NS5A-NS5B junction site (Fig. 1B, P$_{H/B}$). In this chimeric construct, as guided by the crystal structure of NS3-NS4A complexes (23, 50), the NS4A cofactor peptide (GSVVIVGRIVLS) was covalently linked to the N terminus of the HCV NS3 protease catalytic domain (amino acids 3 to 181) through a flexible "GSGS" spacer (43). It has been shown that this N-terminal tethered "single-chain" NS3 represents the activated form of the HCV protease with improved stability and solubility (34, 43). The proximity of the NS4A peptide to the N terminus of the protease allows tighter intercalation and proper folding of the protease domain mimicking that in the "two-chain" NS3-NS4A complexes (23, 50, 51). Due to this economic coupling of the NS3 protease and NS4A cofactor, a much smaller gene was used to replace the Npro coding region, which may have greatly enhanced the genome stability of the resulting chimeric viruses compared to those containing the full-length NS3-NS4A genes (14, 18). Among other functions, Npro is believed to free the BVDV core protein for genome encapsidation and capsid assembly by autoproteolysis of its C terminus. To mimic this function, an NS5A-NS5B junction site (P10-P4') was engineered to link the NS4A-tethered NS3 protease to the BVDV core. This linkage, once cleaved by the N-terminal HCV NS3 protease, would release the BVDV core
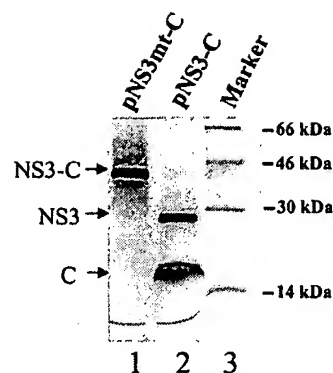

FIG. 2. In vitro expression of the HCV NS3-BVDV core fusion proteins using an in vitro transcription and translation system. The proteins were labeled with [^{35}S]methionine. Lane 1, production of the fusion protein with a catalytically inactive HCV NS3 protease from plasmid pNS3mt-C; lane 2, expression of the fusion protein with the wild-type HCV NS3 protease from plasmid pNS3-C plasmid. The protein products were separated by SDS-PAGE on a 10 to 20% gradient gel and visualized by autoradiography. The fusion protein with the active HCV NS3 protease self-cleaved at the NS5A-NS5B junction between HCV NS3 and BVDV core protein. Lane 3, ^{14}C-labeled molecular mass markers (kilodaltons).

with an additional tetrapeptide "SMSY" at its N terminus (Fig. 1B). As a control, a similar construct, P$_{H/B(S139A)}$, was created with an inactive HCV NS3 protease in which the catalytically essential serine 139 was mutated to alanine (Fig. 1C). This mutation should render the BVDV core unable to be released from the C terminus of the HCV protease domain, resulting in a fusion protein that may not be able to perform its related functions in capsid assembly and virion production. In order to further assess the stability of the chimeric viruses and to address whether the NS4A-tethered HCV NS3 protease can be deleted from the viral genome, an Npro-null BVDV construct (BVDV–Npro) was created (Fig. 1D). In this construct, the entire Npro coding region was removed, and the core protein was directly fused to the start codon (methionine) of the polyprotein.

Feasibility analysis of the chimeric constructs by in vitro translation cleavage assay. To demonstrate whether the newly created chimeric fusion protein would cleave itself as designed to release the BVDV core protein, we cloned the HCV-NS3/BVDV core cDNA (containing the coding regions of the NS4A-tethered HCV NS3 protease plus the core of BVDV) into the pET expression plasmid. The fusion proteins were produced and labeled in rabbit reticulocyte lysate using the in vitro transcription and translation system. The labeled protein products were separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and analyzed as described in Materials and Methods. As shown in Fig. 2, the fusion protein that contains the active HCV NS3 protease domain (pNS3-C) cleaved itself rapidly and yielded two smaller products corresponding to the predicted sizes for HCV NS4A-tethered NS3 and BVDV core (Fig. 2, lane 2). In contrast, the construct with the mutant HCV NS3 protease (pNS3mt-C) failed to be processed and remained as a single and larger fusion processor (Fig. 2, lane 1). These results confirmed that the designed chimeric fusion protein was functional and processed the NS5A-NS5B cleavage site correctly during expression of the polyprotein, indicating that the N-terminal HCV NS3 protease would substitute the proteolytic function of Npro in the polyprotein processing during BVDV infection.
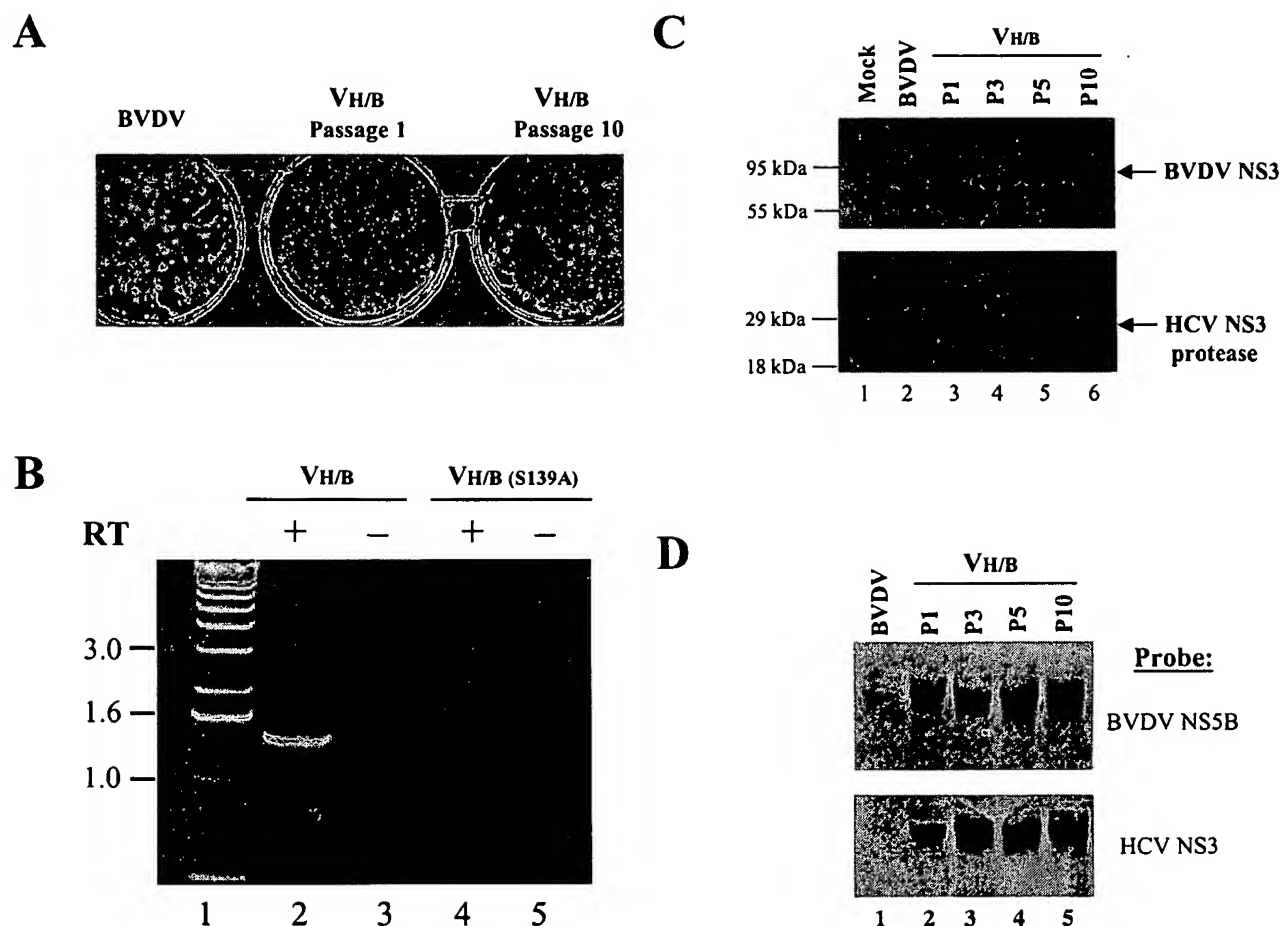
A

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C

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FIG. 3. Characterization of chimeric viruses $V_{H/B}$ obtained from serial passages. (A) Plaque phenotypes. The wild-type BVDV and the chimeric viruses ($V_{H/B}$) from different passages were compared. MDBK cells were infected with BVDV and $V_{H/B}$ from the first passage (P1) and the tenth passage (P10) for 3 days at 37°C. The plaque formation were revealed by crystal violet staining of the MDBK cell monolayer. Plaques formed by the wild-type BVDV (left panel) appeared to be larger in size compared with that formed by the P1 $V_{H/B}$ viruses (central panel). After nine serial passages, P10 $V_{H/B}$ viruses generated larger plaques (right panel). (B) RT-PCR analysis. Viral RNAs were isolated from the third passages of both the chimeric viruses ($V_{H/B}$, lanes 2 and 3) with active HCV NS3 protease and the mutant chimeric viruses ($V_{H/B(S139A)}$, lanes 3 and 4). The HCV NS3-BVDV core coding region in the viral RNAs was amplified in the presence (lanes 2 and 4) or absence (lanes 3 and 5) of reverse transcriptase (RT). DNA molecular size standards (1-kb ladder) is shown in lane 1. (C) Western blot analysis. Cell lysates from mock-infected MDBK cells (lane 1) or from MDBK cells infected by the wild-type BVDV (lane 2) and the chimeric viruses $V_{H/B}$ of various passages (P1, lane 3; P3, lane 4; P5, lane 5; and P10, lane 6). The cell lysates were fractionated by SDS-PAGE on a 10 to 20% gel, transferred to nitrocellulose membrane, and analyzed by immunoblotting with a monoclonal antibody against BVDV NS3 (upper panel) and the polyclonal antibody against HCV NS3 (lower panel). The molecular mass standards (in kilodaltons) are indicated at the left side of each panel. (D) Northern blot analysis. Total RNA was isolated from cells infected by the wild-type BVDV (lane 1) or by the chimeric viruses $V_{H/B}$ of various passages (P1, lane 3; P3, lane 4; P5, lane 5; and P10, lane 6). Glyoxal-denatured viral RNAs were separated by 1% agarose gel electrophoresis and transferred to a nylon membrane. The blot was incubated in the hybridization solution with psoralen-biotinylated DNA probes derived from the BVDV NS5B (upper panel) and the HCV NS3 protease (lower panel). Bands on the blot were visualized by using the BrightStar BioDetect detection kit from Ambion.

Generation of an HCV NS3 protease-dependent BVDV chimeric virus. Having shown that the HCV NS3 protease was processed correctly in the context of the chimeric setting, we built two full-length chimeric clones, $P_{H/B}$ and $P_{H/B(S139A)}$, from the infectious clone of BVDV (isolate NADL) as described previously (46) (also refer to Fig. 1). Full-length RNA transcripts were produced by T7 RNA polymerase in vitro and transfected into MDBK cells by electroporation. Production of viable viruses was examined microscopically for virus-induced CPE and visualized by standard BVDV plaque assay. As shown in Fig. 3A (middle panel), the RNA transcript from the $P_{H/B}$ clone produced viable chimeric viruses ($V_{H/B}$) that formed somewhat smaller plaques (compared to the plaques from the wild-type BVDV in the left panel) on an MDBK cell monolayer 2 to 3 days posttransfection. As expected, the RNA transcript from mutant chimeric clone, $P_{H/B(S139A)}$, failed to pro-

duce any signs of infection. Extracellular supernatant or medium from each cell culture was collected and used to infect naive MDBK cells. After a series of reinfection or passages in fresh MDBK cells, the chimeric viruses from the $P_{H/B}$ clone exhibited larger plaque phenotype (Fig. 3A, right panel), suggesting that viral adaptation or revertant mutation might have occurred. No CPE or plaque was observed in cells inoculated with supernatants derived from the mutant chimeric RNA transfection (data not shown). This was most likely due to the fact that the mutant HCV NS3 protease failed to cleave the NS5A-NS5B junction, preventing the BVDV core from being released from the fusion protein to support virus growth. These data also confirmed that the BVDV NS3 could not substitute HCV NS3 to cleave the HCV NS5A-NS5B junction. Taken together, these results support the idea that the chi-

meric BVDV depends on HCV NS3 protease activity for replication and growth.

The chimeric viruses from the third-passage inoculum (P3 $V_{H/B}$) were further purified on a sucrose gradient. Viral RNA was extracted, and RT-PCR was performed to isolate the cDNA fragment that encompassed the HCV protease and BVDV core fusion (~1.4 kb). The results in Fig. 3B demonstrated that the expected RT-PCR product was only detected in the supernatant from $V_{H/B}$ (Fig. 3B, lane 2) and not from the supernatant of the S139A mutant chimeric RNA (Fig. 3B, lane 4). As a control, PCR alone (without RT) did not produce any products, confirming that the PCR product originated from viral RNA and not from any contaminating DNA sources. The presence of the NS4A-tethered NS3 protease and the HCV NS5A-NS5B junction in the genome of the chimeric viruses was further authenticated by direct sequencing of the RT-PCR products (see Fig. 5). In addition, no BVDV Npro sequence was detected in any of the viral RNA samples (data not shown). This further suggests that the viruses from the chimeric RNA transfection were not due to contaminating cytopathic BVDV (strain NADL).

Genome stability of the chimeric virus. The plaque-purified chimeric viruses (passage 1) were amplified through 10 serial passages up to 10 times by reinfection of fresh MDBK cells. The infectivity of the chimeric viruses was analyzed by microscopic evaluation of CPE or plaque assay. The size of the plaques was larger at later passages than that at passage 1 (Fig. 3A, compare middle and right panels), a size comparable to that of the wild-type BVDV (isolate NADL). To assess the genome stability and to determine whether the HCV NS3 protease was deleted during serial passages of the chimeric viruses, the infected cells at different passages were subjected to Western blot (Fig. 3C) and Northern blot (Fig. 3D) analyses. The results from the Western blot analysis demonstrated the presence of HCV NS3 protease (recognized by the rabbit polyclonal antibodies raised against a purified HCV NS3 protease) throughout all passages of the chimeric viruses (Fig. 3C, lower panel, lanes 3 to 6). The cells infected by the wild-type BVDV did not express the HCV NS3 protease (lane 2). Meanwhile, all infected cells produced the BVDV NS3 and NS2-3 proteins detected by antibodies reactive to BVDV NS3 (Fig. 3C, upper panel). The presence of abundant BVDV NS3 compared to NS2-3 correlated with the observed CPE of the cells infected with the chimeric viruses (Fig. 3C, upper panel, lanes 3 to 6). The Northern blot analysis (Fig. 3D) revealed similar results in that the probe derived from BVDV NS5B hybridized with intracellular viral RNAs from both the wild-type BVDV and the chimeric viruses (upper panel), whereas the probe derived from the HCV NS3 only detected viral RNAs from cells infected by the chimeric viruses (lower panel). The detection of HCV NS3 protease in infected cells as well as its sequence in viral genome RNA following multiple serial passages supports the hypothesis that the chimeric virus are stable, further confirming the dependence of the chimeric viruses on the HCV NS3 protease activity.

One-step viral growth kinetic analysis. Because Npro is dispensable for BVDV replication, it seemed likely that the chimeric virus could lose the tethered HCV NS3 protease, resulting in a Npro-null BVDV. This deletion may occur rapidly if the Npro-null BVDV has certain growth advantages. To better understand the observed stability of the chimeric viruses, an Npro-null virus, BVDV−Npro, was constructed (Fig. 1D). Surprisingly, BVDV−Npro retained the cytopathogenic phenotype of BVDV, although to a lesser extent, and formed smaller plaques on the MDBK cell monolayer. One-step growth kinetic analysis was performed to compare the viral
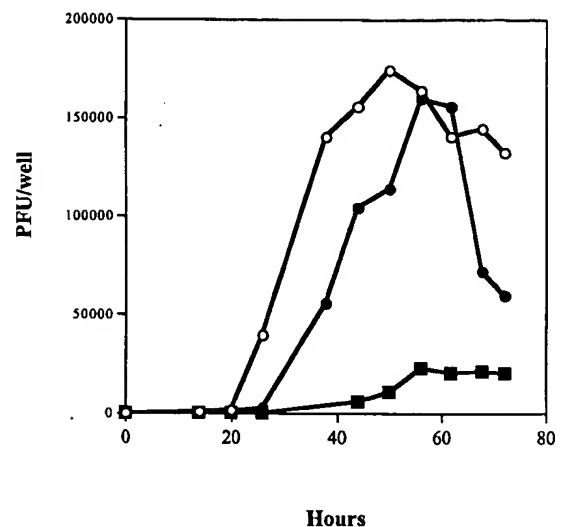

FIG. 4. Single cycle growth kinetic analysis. A total of 20,000 MDBK cells in each well of a 24-well dish were infected by 100,000 PFU of wild-type BVDV (O), chimeric BVDV (●), and BVDV−Npro (■). After 1 h of incubation at 4°C, the inocula were removed, and the cell monolayers were washed thoroughly to remove any residual viruses. Then, 0.5 ml of fresh EMEM with 10% HS was added, and the dish was incubated at 37°C. Newly secreted BVDVs in the media were harvested at various time points postinfection. The virus titers were determined and plotted against time to generate the growth curves.

growth rates, eclipse phases of replication, and the maximum viral yields among wild-type BVDV, chimeric BVDV, and Npro-null BVDV. The results presented in Fig. 4 demonstrated that despite a slight delay of ca. 5 h, the chimeric viruses reached a similar replication efficiency, as reflected by the similar maximum viral yields compared to the wild-type BVDV. In contrast, the Npro-null BVDV had a prolonged eclipse phase and achieved a viral yield at a greatly reduced level, one at least 10 times lower than those of the chimeric and wild-type BVDVs. This suggests that although cytopathic, the propagation of the Npro-null BVDV is greatly compromised, a finding consistent with the hypothesis that the stability of the chimeric viruses is a consequence of the reduced fitness of the potential deletion mutants. Thus far, we have not been able to isolate any Npro-null BVDV-like viruses from chimeric virus-infected cells under optimal viral infection conditions.

Isolation of variant viruses and evidence of viral adaptation. Genomic RNAs were isolated from chimeric viruses after different numbers of passages. The region corresponding to NS4A-tethered HCV NS3 protease plus the NS5A-NS5B junction was amplified by RT-PCR and sequenced directly. The sequences from different passages were compared with the template sequence ($T_{H/B}$) shown in the multiple alignment in Fig. 5. The template sequence was derived from the parent HCV and BVDV clones (43, 46) used to build the chimeric plasmid ($P_{H/B}$). As shown in Fig. 5, three PCR mutations were generated during the construction of the chimeric clone ($P_{H/B}$) (compare the sequence of $P_{H/B}$ with that of $T_{H/B}$). Two mutations occurred in the coding region of HCV NS3 protease, F43 (phenylalanine at amino acid position 43 numbered according to the sequence of HCV NS3) to L (leucine) and D112 (aspartic acid at amino acid position 112) to N (asparagine), and one occurred in the N-terminal coding region for Npro, I4 (isoleucine at position 4) to N (asparagine). After two serial passages, one of the mutations (F43L) quickly reverted to the wild-type at P2, suggesting that this mutation is detrimental to

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        HCV NS4A cofactor  ┌─►    HCV NS3 protease (3-181)
T_H/B   MELITNEGSG SVVIVGRIVL SGSGSITAYA QQTRGLLGCK ITSLTGRDKN 50
P_H/B   ---N------ ---------- --------Y- ---------- ----------
P2      ---N------ ---------- --------C- ---------- ----------
P4      ---N------ ---------- --------C- ---------- ----------
P7      ---N------ ---------- --------C- ---------- ----------
P9      ---N------ ---------- --------C- ---------- ----------

                   HCV NS3 protease (3-181)
T_H/B   QVEGEVQIVS TATQTFLATC INGVCWTVYH GAGTRTIASP KGPVIQMYTN 100
P_H/B   ---------- -----L---- ---------- ---------- ----------
P2      ---------- -----F---- ---------- ---------- ----------
P4      ---------- -----F---- ---------- ---------- ----------
P7      ---------- -----F---- ---------- ---------- ----------
P9      ---------- -----F---- ---------- ---------- ----------

                   HCV NS3 protease (3-181)
T_H/B   VDQDLVGWPA PQGSRSLTPC TCGSSDLYLV TRHADVIPVR RRGDSRGSLL 150
P_H/B   ---------- ---------- ---------- ----N----- ----------
P2      ---------- ---------- ---------- ----N----- ----------
P4      ---------- ---------- ---------- ----N----- ----------
P7      ---------- ---------- ---------- ----N----- ----------
P9      ---------- ---------- ---------- ----N----- ----------

                   HCV NS3 protease (3-181)
T_H/B   SPRPISYLKG SSGGPLLCPA GHAVGLFRAA VCTRGVAKAV DFIPVENLET 200
P_H/B   ---------- ---------- ---------- ---------- ----------
P2      ---------- ---------- ---------- ---------- ----------
P4      ---------- ---------- ---------- ---------- ----------
P7      ---------- ---------- ---------- ---------- ----------
P9      ---------- ---------- ---------- ---------- ----------

        ◄─┐ HCV 5A/5B junction  ┌─► BVDV Core
T_H/B   TMRSGSGADT EDVVCCSMSY SDTKE 225
P_H/B   -M-------- ---------- -----
P2      -T-------- ---------- -----
P4      -T-------- ---------- -----
P7      -T-------- ---------- -----
P9      -T-------- ---------- -----
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FIG. 5. Amino acid sequence analysis of the HCV NS3 protease coding region in the chimeric viruses at different serial passages. cDNA fragments (1.4 kb) encompassing part of the BVDV 5' UTR, NS4A-tethered HCV NS3 protease, the HCV NS5A-NS5B junction site, the BVDV core, and the E^{rns} were generated by RT-PCR from viral genomic RNAs and cloned into pCR2.1-TOPO vector by using TOPO TA Cloning Kit (Invitrogen). All clones were verified by dideoxynucleotide sequencing. The amino acid sequences encoding the N terminus of Npro, the HCV NS4A cofactor (underlined), the HCV NS3 protease domain (boldface type), the HCV 5A-5B junction site (underlined), and the N terminus of BVDV core are compared. T$_{H/B}$ represents the template sequences for all fusion components derived from their respective parent clones (43, 46). P$_{H/B}$ represents the starting chimeric BVDV clone used to generate infectious RNA transcripts and transfect MDBK cells. The sequences from chimeric BVDV at different passages (P$_{H/B}$, passage 0; P2, passage 2; P4, passage 4; P7, passage 7; P9, passage 9) were compared and aligned with the lead sequence of T$_{H/B}$. -, Identical amino acid compared to the corresponding one in T$_{H/B}$.
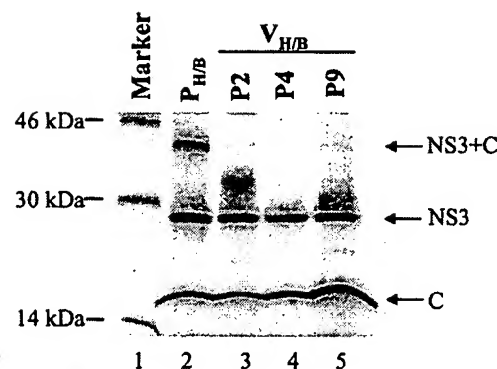

FIG. 6. Correlation of the HCV NS3 protease activity with viral adaptation. The coding regions for the HCV NS3 protease and BVDV core were isolated either from plasmid P$_{H/B}$ by PCR or from the chimeric viruses V$_{H/B}$ at various passages (P2, P4, and P9) by RT-PCR. These coding regions were subcloned into the pET-28a expression vector (see Materials and Methods). The fusion proteins were expressed and labeled with [^{35}S]methionine by using the in vitro transcription and translation system. The labeled fusion precursor and the cleavage products were separated by SDS-PAGE and detected by autoradiography. Lane 1 indicates the ^{14}C-labeled molecular mass marker (in kilodaltons).

the chimeric viruses. Interestingly, two additional point mutations were identified in the HCV NS3 coding region from the chimeric viruses of later passages: Y6 (tyrosine at amino acid position 6) to C (cysteine) and M179 (methionine at position 179) to T (threonine). Both mutations were near the chimeric junctions, which might allow better folding of the fusion protein.

To correlate these mutations or variations with the protease activity, the cDNA fragments encompassing the HCV NS3 protease and BVDV core fusion protein were cloned into the pET expression plasmid as described for Fig. 2. Results from the in vitro transcription and translation cleavage assay demonstrated that the starting chimeric clone (P$_{H/B}$) harboring the F43L mutation exhibited reduced protease activity (Fig. 6, lane 2), as reflected by the incomplete cleavage of the fusion precursor. The fusion proteins from the later passages (P2 to P9) had normal levels of the protease activity comparable to that of the wild-type construct (pNS3-C, equivalent of T$_{H/B}$, in Fig. 2, lane 2). These data suggest that there is a correlation between viral adaptation and enhancement of HCV protease activity. Studies by others using the Sindbis chimeric viruses also re-

ported a similar point mutation (C16Y) without a clear correlation with the in vitro protease activity (14). This is consistent with our observations that some of the mutations or variations (Y6C and M179T) do not correlate with any detectable changes in the protease activity (compare Fig. 2 and 6). Other mutations (I4N in Npro and D112N in HCV NS3) can be tolerated and sustained throughout serial passages. We have not observed any deletions in the HCV NS3 region, as reported by others who used the full-length NS3-NS4A for the construction of chimeric viruses (14), possibly due to the economic packing of the minimum NS4A cofactor and the small catalytic domain of HCV NS3 protease in our construct.

DISCUSSION

The lack of a cell culture system that permits HCV infection imposes a major obstacle for anti-HCV drug development. Although substantial efforts have been devoted to the characterization of HCV NS3 protease (23, 50), the inhibitor development against HCV NS3 protease is limited without a good cell-based system to evaluate its cellular potency. Several cell-based *trans*-cleavage assays have been developed relying on the coexpression of an NS3 protease-containing plasmid and a substrate-containing plasmid in the same cells (5, 20). However, these systems often yield less-reproducible results and, more importantly, fail to reflect the polyprotein processing which occurs within the subcellular microenvironment of viral replication. Such intrapolyprotein processing may resemble the kinetics of a *cis*-cleavage which is insensitive to dilution.

Several HCV NS3 protease-dependent chimeric viruses have been created (13, 14, 18) using two plus-stranded RNA viruses from unrelated virus families as the carriers. In these chimeric viruses, HCV NS3 protease-mediated protein processings (mostly *cis*-cleavages) are essential for virus growth. However, these chimeric viruses are less stable because the inserted HCV NS3-NS4A can be easily deleted, resulting in reversion to the wild-type viruses which have many growth advantages over the chimeric viruses (14). In this approach, we took advantage of the dispensability of Npro for BVDV replication and substituted its proteolytic function with that of HCV NS3 protease. The resulting chimeric BVDV was cytopathic and easy to quantify. Its growth properties were com-

parable to those of the wild-type BVDV. Most importantly, under normal cell culture conditions (with a low MOI and without any selection pressures against the chimeric viruses), this HCV protease-dependent BVDV was very stable, lacking any detectable deletions in the HCV coding regions.

One of the key benefits of developing stable chimeric viruses is the possibility to assess potential drug resistance to anti-HCV NS3 protease inhibitors. This will require that the chimeric viruses be very stable. Whether the chimeric viruses reported here were stable enough to allow the selection of drug-resistant variants remains to be addressed. Based on a prediction that the HCV NS3 protease might be deleted to yield a virus without Npro, we created a molecular clone lacking Npro (BVDV–Npro) in which the entire Npro coding region was removed (Fig. 1). To our surprise, the Npro-null BVDV was not only viable but also cytopathic and formed small plaques on a cell monolayer. Further growth analysis revealed that this Npro-null BVDV was highly defective in replication and achieved a virus production level at least 10 times lower than that of the chimeric viruses or the wild-type viruses. While this partially explained the observed stability of the chimeric BVDV, it also limited the chimeric viruses from being used to select potential mutations that conferred drug resistance. Under a strong selection against the chimeric viruses, such as a potent HCV NS3 protease inhibitor, the Npro-null BVDV would appear quickly and may overtake the virus production.

In a cell culture dish, the chimeric BVDV appeared to retain comparable growth properties and CPE as the wild-type virus. Whether the chimeric virus replicates in vivo in calves and duplicate the course of disease progression as the wild-type virus will be an interesting subject to address. A better understanding of the chimeric virus correlated with the Npro-null BVDV will help to elucidate the biological function of this unique Npro protein acquired by BVDV. The one-step growth analysis has revealed that this virus is defective in replication, suggesting that BVDV without the Npro is attenuated. If the in vivo studies demonstrate that the Npro-null BVDV, or even the chimeric virus, is attenuated with self-limiting infection which induces anti-BVDV responses, it may be an excellent candidate for vaccine development. Finally, the HCV NS3 protease-dependent BVDV may provide an alternative and much more affordable animal model for the in vivo testing of any HCV protease inhibitors.

ACKNOWLEDGMENTS

REFERENCES

1. Alter, H. J., R. H. Purcell, J. W. Shih, J. C. Melpolder, M. Houghton, Q.-L. Choo, and G. Kuo. 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A non-B hepatitis. N. Engl. J. Med. 321:1494–1500.
2. Alter, M. J., D. Kruszon-Moran, O. V. Nainan, G. M. McQuillan, F. Gao, L. A. Moyer, R. A. Kaslow, and H. S. Margolis. 1999. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. N. Engl. J. Med. 341:556–562.
3. Bartenschlager, R., L. Ahlborn-Laake, J. Mous, and H. Jacobsen. 1993. Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. J. Virol. 67:3835–3844.
4. Cho, Y. G., H. S. Moon, and Y. C. Sung. 1997. Construction of hepatitis C-SIN virus recombinants with replicative dependency on hepatitis C virus serine protease activity. J. Virol. Methods 65:201–207.
5. Cho, Y. G., S. H. Yang, and Y. C. Sung. 1998. In vivo assay for hepatitis C viral serine protease activity using a secreted protein. J. Virol. Methods 72:109–115.
6. Chon, S. K., D. R. Perez, and R. O. Donis. 1998. Genetic analysis of the internal ribosome entry segment of bovine viral diarrhea virus. Virology 251:370–381.
7. Choo, Q.-L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–364.
8. Collett, M. S., R. Larson, C. Gold, D. Strick, D. K. Anderson, and A. F. Purchio. 1988. Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus. Virology 165:191–199.
9. Davis, G. L., R. Esteban-Mur, V. Rustgi, J. Hoefs, S. C. Gordon, C. Trepo, M. L. Shiffman, S. Zeuzem, A. Craxi, M. H. Ling, and J. Albrecht. 1998. Interferon alpha-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. N. Engl. J. Med. 339:1493–1499.
10. Elbers, K., N. Tautz, P. Becher, D. Stoll, T. Rumenapf, and H.-J. Thiel. 1996. Processing in the pestivirus E2-NS2 region: identification of proteins p7 and E2p7. J. Virol. 70:4131–4135.
11. Failla, C., L. Tomei, and R. De Francesco. 1994. both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. J. Virol. 68:3753–3760.
12. Farci, P., H. J. Alter, S. Govindarajan, D. C. Wong, R. Engle, R. R. Lesniewski, I. K. Mushahwar, S. M. Desai, R. H. Miller, N. Ogata, and R. H. Purcell. 1992. Lack of protective immunity against reinfection with hepatitis C virus. Science 258:135–140.
13. Filocamo, G., L. Pacini, and G. Migliaccio. 1997. Chimeric Sindbis viruses dependent on the NS3 protease of hepatitis C virus. J. Virol. 71:1417–1427.
14. Filocamo, G., L. Pacini, C. Nardi, L. Bartholomew, M. Scaturro, P. Delmastro, A. Tramontano, R. De Francesco, and G. Migliaccio. 1999. Selection of functional variants of the NS3-NS4A protease of hepatitis C virus by using chimeric Sindbis viruses. J. Virol. 73:561–575.
15. Frolov, I., M. S. McBride, and C. M. Rice. 1998. cis-acting RNA elements required for replication of bovine viral diarrhea virus-hepatitis C virus 5' nontranslated region chimeras. RNA 4:1418–1435.
16. Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice. 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of the proteinase-dependent polyprotein cleavage sites. J. Virol. 67:2832–2843.
17. Grassmann, C. W., O. Isken, and S.-E. Behrens. 1999. Assignment of the multifunctional NS3 protein of bovine viral diarrhea virus during RNA replication: an in vitro and in vivo study. J. Virol. 73:9196–9205.
18. Hahm, B., S. H. Back, T. G. Lee, E. Wimmer, and S. K. Jang. 1996. Generation of a novel poliovirus with a requirement of hepatitis C virus protease NS3 activity. Virology 226:318–326.
19. Hijikata, M., H. Mizushima, T. Akagi, S. Mori, N. Kakiuchi, N. Kato, T. Tanaka, K. Kimura, and K. Shimotohno. 1993. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J. Virol. 67:4665–4675.
20. Hirowatari, Y., M. Hijikata, and K. Shimotohno. 1995. A novel method for analysis of viral proteinase activity encoded by hepatitis C virus in cultured cells. Anal. Biochem. 225:113–120.
21. Hong, Z., E. Ferrari, J. Wright-Minogue, R. Chase, C. Risano, G. Seelig, C.-G. Lee, and A. D. Kwong. 1996. Enzymatic characterization of hepatitis C virus NS3/4A complexes expressed in mammalian cells using the herpes simplex virus amplicon system. J. Virol. 70:4261–4268.
22. Horton, R. M., Z. L. Cai, S. N. Ho, and L. R. Pease. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. BioTechniques 8:528–535.
23. Kim, J. L., K. A. Morgenstern, C. Lin, T. Fox, M. D. Dwyer, J. A. Landro, S. P. Chambers, W. Markland, C. A. Lepre, E. T. O'Malley, S. L. Harbeson, C. M. Rice, M. A. Murcko, P. R. Caron, and J. A. Thomson. 1996. Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. Cell 87:343–355.
24. Kwong, A. D., J. L. Kim, G. Rao, D. Lipovsek, and S. A. Raybuck. 1999. Hepatitis C virus NS3/4A protease. Antiviral Res. 41:67–84.
25. Lin, C., J. A. Thomson, and C. M. Rice. 1995. A central region in the hepatitis C virus NS4A protein allows formation of an active NS3-NS4A serine proteinase complex in vivo and in vitro. J. Virol. 69:4373–4380.
26. McHutchison, J. G., S. C. Gordon, E. R. Schiff, M. L. Shiffman, W. M. Lee, V. K. Rustgi, Z. D. Goodman, M. H. Ling, S. Cort, and J. K. Albrecht. 1998. Interferon alpha-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N. Engl. J. Med. 339:1485–1492.
27. Mehta, R. K., and J. Singh. 1999. Bridge-overlap-extension PCR method for constructing chimeric genes. BioTechniques 26:1082–1086.
28. Mendez, E., N. Ruggli, M. S. Collett, and C. M. Rice. 1998. Infectious bovine viral diarrhea virus (strain NADL) RNA from stable cDNA clones: a cellular insert determines NS3 production and viral cytopathogenicity. J. Virol. 72:4737–4745.
29. Meyers, G., and H.-J. Thiel. 1996. Molecular characterization of pestiviruses. Adv. Virus Res. 47:53–118.
30. Mittelholzer, C., C. Moser, J. D. Tratschin, and M. A. Hofmann. 1997. Generation of cytopathogenic subgenomic RNA of classical swine fever virus

in persistently infected porcine cell lines. Virus Res. **51**:125–137.

31. **Moser, C., P. Stettler, J.-D. Tratschin, and M. A. Hofmann.** 1999. Cytopathogenic and noncytopathogenic RNA replicons of classical swine fever virus. J. Virol. **73**:7787–7794.

32. **Moser, C., J. D. Tratschin, and M. A. Hofmann.** 1998. A recombinant classical swine fever virus stably expresses a marker gene. J. Virol. **72**:5318–5322.

33. **Ohba, K., M. Mizokami, J. Y. Lau, E. Orito, K. Ikeo, and T. Gojobori.** 1996. Evolutionary relationship of hepatitis C, pesti-, flavi-, plantviruses, and newly discovered GB hepatitis agents. FEBS Lett. **378**:232–234.

34. **Pasquo, A., M. C. Nardi, N. Dimasi, L. Tomei, C. Steinkuhler, P. Delmastro, A. Tramontano, and R. De Francesco.** 1998. Rational design and functional expression of a constitutively active single-chain NS4A-NS3 proteinase. Fold Des. **3**:433–441.

35. **Poole, T. L., C. Wang, R. A. Popp, L. N. Potgieter, A. Siddiqui, and M. S. Collet.** 1995. Pestivirus translation initiation occurs by internal ribosome entry. Virology **206**:750–754.

36. **Rice, C. M.** 1996. Flaviviridae: the viruses and their replication, p. 931–960. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology, 3rd ed. Raven Press, New York, N.Y.

37. **Rice, C. M., A. Grakoui, R. Galler, and T. J. Chambers.** 1989. Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. New Biol. **1**:285–296.

38. **Rumenapf, T., R. Stark, M. Heimann, and H. J. Thiel.** 1998. N-terminal protease of pestiviruses: identification of putative catalytic residues by site-directed mutagenesis. J. Virol. **72**:2544–2547.

39. **Senanayake, S. D., and D. A. Brian.** 1995. Precise large deletions by the PCR-based overlap extension method. Mol. Biotechnol. **4**:13–15.

40. **Shimizu, Y. K., M. Hijikata, A. Iwamoto, H. J. Alter, R. H. Purcell, and H. Yoshikura.** 1994. Neutralizing antibodies against hepatitis C virus and the emergence of neutralization escape mutant viruses. J. Virol. **68**:1494–1500.

41. **Stark, R., G. Meyers, T. Rumenapf, and H. J. Thiel.** 1993. Processing of pestivirus polyprotein: cleavage site between autoprotease and nucleocapsid protein of classical swine fever virus. J. Virol. **67**:7088–7095.

42. **Tanji, Y., M. Hijikata, S. Satoh, T. Kaneko, and K. Shimotohno.** 1995. Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. J. Virol. **69**:1575–1581.

43. **Taremi, S. S., B. Beyer, M. Maher, N. Yao, W. Prosise, P. C. Weber, and B. A. Malcolm.** 1998. Construction, expression, and characterization of a novel fully activated recombinant single-chain hepatitis C virus protease. Protein Sci. **7**:2143–2149.

44. **Tautz, N., T. Harada, A. Kaiser, G. Rinck, S.-E. Behrens, and H.-J. Thiel.** 1999. Establishment and characterization of cytopathogenic and noncytopathogenic pestivirus replicons. J. Virol. **73**:9422–9432.

45. **Tratschin, J. D., C. Moser, N. Ruggli, and M. A. Hofmann.** 1998. Classical swine fever virus leader proteinase Npro is not required for viral replication in cell culture. J. Virol. **72**:7681–7684.

46. **Vassilev, V. B., M. S. Collett, and R. O. Donis.** 1997. Authentic and chimeric full-length genomic cDNA clones of bovine viral diarrhea virus that yield infectious transcripts. J. Virol. **71**:471–478.

47. **Wimmer, E., C. U. Hellen, and X. Cao.** 1993. Genetics of poliovirus. Annu. Rev. Genet. **27**:353–436.

48. **Wyatt, C. A., L. Andrus, B. Brotman, F. Huang, D.-H. Lee, and A. M. Prince.** 1998. Immunity in chimpanzees chronically infected with hepatitis C virus: role of minor quasispecies in reinfection. J. Virol. **72**:1725–1730.

49. **Xu, J., E. Mendez, P. R. Caron, C. Lin, M. A. Murcko, M. S. Collett, and C. M. Rice.** 1997. Bovine viral diarrhea virus NS3 serine proteinase: polyprotein cleavage sites, cofactor requirements, and molecular model of an enzyme essential for pestivirus replication. J. Virol. **71**:5312–5322.

50. **Yan, Y., Y. Li, S. Munshi, V. Sardana, J. L. Cole, M. Sardana, C. Steinkuehler, L. Tomei, R. De Francesco, L. C. Kuo, and Z. Chen.** 1998. Complex of NS3 protease and NS4A peptide of BK strain hepatitis C virus: a 2.2 Å resolution structure in a hexagonal crystal form. Protein Sci. **7**:837–847.

51. **Yao, N., P. Reichert, S. Taremi, W. W. Prosise, and P. C. Weber.** 1999. Molecular views of viral polyprotein processing revealed by the crystal structure of the hepatitis C virus bifunctional protease-helicase. Structure **7**:1353–1363.